Molecular epidemiology of tuberculosis in Vietnam

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Financial support for the publication of this thesis was obtained from the Department of Pulmonary Diseases and Medical Microbiology, Radboud University Nijmegen Medical Centre, Nijmegen, and from KNCV Tuberculosis Foundation, The Netherlands.

Cover design by IS Ontwerp: Ilse Schrauwers, The Netherlands (www.isontwerp.nl) with photograph by Mai Nguyệt Thu Huyền and map designed by Tôn Đức Thọ.
Layout of thesis by Mai Nguyệt Thu Huyền

Printing: Ipskamp drukkers, Enschede, The Netherlands (www.ipskampdrukkers.nl)

ISBN: xxx

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Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
in het openbaar te verdedigen op maandag 3 februari 2014
om 14.30 uur precies

door

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geboren op 4 november 1967
te Ho Chi Minh Stad, Vietnam

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Doctoral Thesis

to obtain the degree of doctor
from Radboud University Nijmegen
on the authority of the Rector Magnificus prof. dr. S.C.J.J. Kortmann,
according to the decision of the Council of Deans
to be defended in public on Monday, February 3, 2014
at 14.30 hours

by

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Contents

CHAPTER 1

GENERAL INTRODUCTION

I. History of tuberculosis

Tuberculosis has been a disease of mankind since ancient times but for long, its cause remained unknown. In 460 BC, Hippocrates defined phthisis as a contagious disease often seen in young people with symptoms such as fever in the afternoon, fierce cough, poor appetite, and high mortality [1].

In the 17th century, due to the crowded living conditions and poor hygiene in the expanding cities tuberculosis became epidemic in Europe, and referred to as the Great White Plague – an epidemic that started abating only after 200 years.

Tuberculosis epidemic then spread in Africa with an alarming morbidity and mortality rate of 700/100.000 population in 1912 [2] when the slaves brought the disease back home from Europe. Only until 1882, the hidden killer causing death to millions of people around the world was found by Robert Koch. Thanks to his research, the tubercle bacillus, the cause of one in seven deaths in the mid-19th century was identified, and a new method was developed to grow *Mycobacterium tuberculosis* on cultures by using solid media made of potatoes and agar. This formed the scientific basis for future bio-molecular research into *Mycobacterium tuberculosis* [1].

II. Burden and epidemiology of tuberculosis worldwide and in Vietnam

Tuberculosis (TB) is still a burden all over the world, especially in developing countries. Worldwide, every second there is 1 person infected with tuberculosis and one third of population is infected with this disease. 5-10% of TB-infected people will become diseased with tuberculosis during their life [3]. Without treatment, 70% of smear-positive TB patients and 20% of smear-negative but culture-positive TB patients die within 10 years [4]. Since anti-TB drugs have been used, the mortality rate of TB dramatically decreased, and cure rates of more than 90% have been recorded in clinical trials. Nevertheless, TB remains a major global health problem. In 1993, the World Health Organization (WHO) estimated 7–8 million cases and 1.3–1.6 million deaths occurred each year and proclaimed TB a global public health emergency [5]. HIV/AIDS has badly impacted on TB control [1]. Countries in Sub-Saharan Africa that have high prevalence of HIV have recorded TB incidence rates increasing two or three fold in the 1990s [6].

According to the WHO, there were in 2011 about 8.7 million people infected with *Mycobacterium tuberculosis* (MTB), 13% of whom were co-infected with HIV, and about 1.4 million TB deaths around the world. Most of TB cases were notified in Asia (59%) and Africa (29%), and TB is the leading cause of adult deaths from an infectious disease after HIV, especially in women [7].

In 2006, with a prevalence of smear-positive tuberculosis of 89 per 100,000 population, Vietnam ranked 12 among the 22 countries with the highest burden of TB in the world, and ranked third in the Pacific Ocean – East Asia region after China and the Philippines (WHO, unpublished data, 2008). Hoa *et al*. conducted a TB prevalence survey in 2007, revealing that the prevalence rate of smear-positive tuberculosis was 145 per 100,000 (95% CI: 110–180), which is 1.6 times higher than previously estimated [8], and higher in South Vietnam than in the Northern and Central parts of the

country. WHO estimated that in 2011, there were around 323,000 prevalent cases of TB (all forms) (95%CI: 148,000-563,000) in the whole of Vietnam [7].

The MTB Beijing genotype, which is one of the common genotypes in Vietnam, was estimated to be responsible for 35% of the TB cases in a rural area of Vietnam, and associated with young age and multidrug resistant (MDR) TB [9]. It is unclear whether this genotype is also associated with unfavorable treatment outcomes such as treatment failure or relapse, or with specific drug resistance-conferring mutations, and whether variable number of tandem repeats (VNTR) typing is as good as restriction fragment polymorphism (RFLP) typing in discriminating among strains of the Beijing genotype. These questions will be addressed in this thesis.

III. Multidrug resistant tuberculosis

Multidrug resistance worldwide

Besides HIV co-infection, TB drug resistance has been threatening TB management in many countries around the world [10]. Generally considered a man-made problem [11], the incidence of drug resistant TB has reached a record level and is becoming a serious threat to global health, with an estimated number of MDR cases (i.e., resistant to both rifampicin and isoniazid) in 2011 of 630.000 (460.000-790.000) among 12 million cases of TB worldwide [7]. In the hope of eliminating TB in the near future, the WHO has in its Global Plan to Stop TB 2011–2015 included the objectives that:

- By 2015, all previous treated patients should be tested for MDR-TB
- Any new TB patients, who are likely at high risk of MDR-TB (for example, those exposed to MDR-TB patients or living in settings with high prevalence of MDR-TB) should also be tested for MDR-TB using rapid tests by 2015, and
- In recognition of the availability of rapid tests, more than 50% of tests for drug resistance among new TB patients and more than 90% of tests among previously treated patients should be done using rapid tests by 2015.
- Among diagnosed cases of MDR-TB, at least 90% should have drug susceptibility results for a fluoroquinolone and an injectable drug (aminoglycoside, polypeptide), which are the mainstay of second-line treatment [12].

Tuberculosis treatment and drug resistance in Vietnam

From 1975 to 1988, two common regimens in Vietnam were 3 months of streptomycin (S), isoniazid (H) and pyrazinamide (Z) daily followed by 6 months of S and H biweekly (3SHZ/6S2H2) and 3 months of rifampicin (R), H and Z daily, followed by 6 months of R, H and ethambutol (E) biweekly (3RHZ/6R2H2E2), applied for new and previously treated TB patients, respectively.

In 1981, a survey in 11 provinces in the Cuulong river delta showed a prevalence of streptomycin resistance of 64% that was slightly lower than in the North of Vietnam (67%) (reported by Dr. Tran Tan Quang, unpublished data).

Directly Observed Treatment Short-course (DOTS), the core of the global strategy to control TB, was introduced in Vietnam in 1986, and short-course

chemotherapy (2SHRZ/6HE) was introduced for treatment new TB patients in 1989. DOTS coverage achieved 100% by 1997, and since then the WHO target for treatment success for new smear-positive cases has been met [13,14].

In 2001, a survey of drug resistance reported among new TB patients the prevalence of resistance to any drug to be 26.3%, to isoniazid 16.6%, to rifampin 2.0%, to ethambutol 1.1%, to streptomycin 19.4%. Among previously treated patients the prevalence of any resistance was 62.9%, and of MDR 23.2%.[15]. Furthermore 80% of patients with a history of multiple first-line treatment courses (so called chronic TB patients) had MDR-TB [16]. In WHO drug resistance report 2010, Vietnam was one of 27 high MDR-TB burden countries, with 2.7% (2–3.6) MDR-TB in new TB cases and 19% (14.5–25.2) in previously treated TB cases [17].

Drug resistance mechanisms

Rifampicin and isoniazid are two important drugs for first-line anti-tuberculosis treatment [18]. Whereas rifampicin resistance is usually coded in the core region of the *rpoB* gene, the mechanism of the resistance to isoniazid is quite complex with mutations conferring resistance located in several loci and genes (*katG*, *inhA*, *ahpC*, and, potentially, also *ndh*) [19-22]. Of the latter, mutations in codon 315 of the *katG* gene and in the promoter region of the *inhA* gene are the most common, occurring in 50-100% of isoniazid resistant strains [21, 23]. Associations between *katG* codon 315 mutations and MDR have been reported in several studies [24-26], and some suggested that these mutations were more frequent in clinical Beijing genotype strains [24]. Furthermore, population studies have suggested that strains with mutations in the *katG* 315 codon or the *inhA* promoter region are more likely to spread than strains with other mutations [27, 28].

The genome of *Mycobacterium tuberculosis* contains many different regions including IS*6110*, a direct repeat locus which contains 10 to 50 copies of a 36-bp direct repeat, separated from one another by spacers that have different sequences, as well as many mycobacterial interspersed repeat units (MIRUs) [29]. Based on these specific regions several molecular techniques were developed for diagnosis of TB (IS*6110* PCR) or molecular typing of MTB (RFLP, spoligotyping, and VNTR).

IV-Molecular methods for diagnosis and typing of tuberculosis

Molecular techniques for diagnosis

Since in 1985 the polymerase chain reaction (PCR) was invented by Kary Mullis [30], many kinds of PCR methods have been applied to detect MTB in clinical samples containing very few MTB organisms that cannot be detected by microscopic examination of Ziehl–Neelsen stained smears. The most notable was IS*6110* PCR that has been applied in TB diagnosis in Vietnam since the 1990s.

As mentioned above, drug resistance especially MDR-TB is a serious problem that hampers the success of TB control worldwide. To cope with this problem, drug resistance patterns should be available as soon as possible to guide the therapy for MDR-TB patients. However, phenotypic drug susceptibility testing (DST) is a time-consuming process because it requires culturing, which may take up to two months or

longer. Recently molecular drug resistance testing techniques were introduced such as the INNO-LiPA Rif.TB assay (rapid detection of rifampicin resistance) and the GenoType® MTBDR*plus* assay (rapid detection of rifampicin and isoniazid resistance). Their principle is a line-probe assay for the detection of *M. tuberculosis* complex and specific mutations in specific genes conferring drug resistance. The turnaround time is 24 hours, and because of limited sensitivity with low bacterial load these methods are applied only for smear-positive samples.

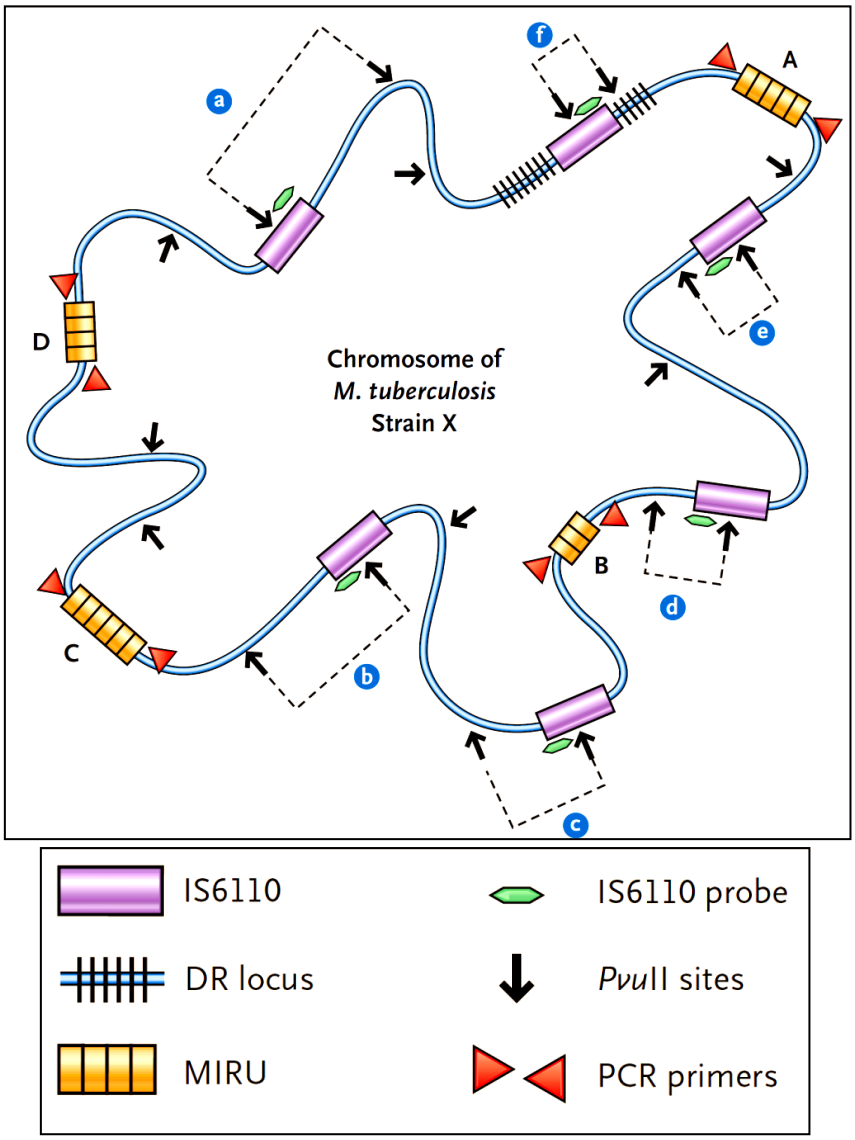

Figure: *Mycobacterium tuberculosis* genome. Reproduced with permission from [29]; copyright Massachusetts Medical Society.

The GenoType® MTBDR*plus* assay, introduced in 2004, identifies *M. tuberculosis* complex and simultaneously detects mutations in the *rpoB* gene as well as mutations in the *katG* gene (associated with high-level isoniazid resistance) and *inhA* promoter region mutations (associated with low-level isoniazid resistance) [31].

Late 2009 GeneXpert MTB/RIF® was introduced, an assay that marked an obvious advance in molecular diagnosis of TB. GeneXpert is a cartridge-based real time PCR test for detecting MTB and screening for rifampicin resistance with high sensitivity even in smear-negative samples. With a turnaround time of less than 2 hours, it is easy to perform and has no requirement for skilled technicians [32].

Confronted with the situation of MDR-TB in the world, in June 2008 WHO endorsed the use of molecular line-probe assays for MDR-TB screening and the GenoType® MTBDR*plus* assay has since been introduced for routine practice in various countries [33-38]. WHO recommends that before using the assay in routine TB treatment and control, the performance of the assay in relation to the locally circulating *M. tuberculosis* bacteria should be validated [39]. As Vietnam is one of the 27 high MDR-TB burden countries [7], application of rapid DST detecting MDR-TB in Vietnam is necessary.

Molecular techniques for typing

Since the 1990s restriction fragment length polymorphism (RFLP) typing has been used, based on a difference in homologous DNA sequences that can be detected by the presence of IS*6110* fragments of different lengths in MTB genome. A labeled IS*6110* probe is hybridized with one or more target fragments after digestion by specific restriction endonucleases (PVU II), separated by electrophoresis and transferred onto a nylon membrane, thus revealing a unique blotting pattern characteristic to a specific genotype at a specific locus [40]. RFLP**,** as a molecular marker, was until recently considered the gold standard typing method in the molecular epidemiology of tuberculosis [41]. This method has been used to track TB outbreaks [42], to confirm recurrence of TB due to reactivation of the same strain or to re-infection with different strains [43], and to identify misdiagnosis due to laboratory cross-contamination [44, 45].

Although RFLP revealed a high level of discrimination among MTB isolates, it was considered complicated, technically demanding, and time consuming. Moreover this technique cannot discriminate strains containing fewer than 5 copies of IS*6110* (low copy strains) and the complex RFLP patterns are difficult to interpret and exchange.

In recent years, spoligotyping has become one of the most widely used genotyping methods for epidemiological studies of TB and for classification of MTB genotype families [46]. This method is based on PCR, amplifying the whole Direct Repeat (DR) region with a biotinylated specific primer. The biotin-labeled PCR products are hybridized to the immobilized spacer-oligos that represent spacers of known sequence. The presence of spacers is then visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection (http://www.moleculartb.org /gb/pdf/protocols/SpoligoManual.pdf) [47].

In comparison to RFLP, spoligotyping provides some important advantages: simplicity, rapidity, high reproducibility and stability of the results. However, its discriminatory power is much lower. Hence spoligotyping is only useful for

classification of MTB strain families causing outbreaks, whereas another method (such as RFLP) needs to be used to track transmission between individual patients.

The new generation genotyping of *M. tuberculosis* based on the detection of the variable number of tandem repeats (VNTRs) of mycobacterial interspersed repetitive units (MIRUs) is considered the next gold standard in the molecular epidemiology of tuberculosis [48]. VNTR or MIRU typing is also based on PCR amplification of multiple genomic loci to determine the number of tandem repeats at these sites. This typing is much easier than IS*6110*-RFLP typing, and is applicable to crude low-concentration DNA extracts. Moreover, the results are expressed as numerical codes and are, therefore, easy to compare and exchange. Several studies indicated that VNTR typing is as discriminative as the RFLP typing method in discrimination of *M. tuberculosis* with multi-banded IS*6110* RFLP [49, 50].

V-Molecular epidemiology of tuberculosis

Molecular epidemiology can be used to determine the distribution of genotypes in different regions in the world, track outbreaks of TB, and distinguish recurrent TB disease due to relapses from that due to re-infection. It also allows studying possible associations between genotype and treatment outcomes, drug resistance, or other patient factors, and thereby can help identify reasons for treatment failure and relapse that affect the control of TB in specific regions or in the world in general. Based on such results we can possibly improve TB control by adjusting TB treatment regimens and recommending appropriate policies for TB diagnosis.

Genotypes of tuberculosis

The W-Beijing genotype, first described in 1995, has been encountered in many areas of the world [51-53]. Especially, it is among the predominant genotypes in most of countries of East and Southeast Asia (53, 54) and associated with drug resistance (high level in Cuba, the former Soviet Union, Vietnam, and South Africa) [53].

In Vietnam, 54% of new patients in urban areas were infected with the Beijing genotype, whereas this genotype was isolated in 35% TB patients in the rural South of Vietnam [9, 55]. In both studies the Beijing genotype was predominant in the youngest age groups, and was more common among patients with resistance to isoniazid or streptomycin. In the rural South it was also associated with MDR-TB [9].

Previous studies suggested an association between relapse and Beijing genotype [56-58]. In a small study Lan *et al*. found that Beijing genotype was related to relapse and/or treatment failure but could not distinguish between the two [56]. A recent study in Indonesia suggested that *M. tuberculosis* Beijing genotype strains were associated with TB treatment failure, even in the absence of drug resistance [59]. However, it is unclear whether TB relapse is truly associated with genotype in Vietnam.

Besides the Beijing genotype, East African Indian (EAI) strains are also a common genotype in South East Asian [60, 61]. In rural South Vietnam 49% of circulating strains were low copy strains, all belonging to the EAI genotype family [9]. Based on the single-nucleotide polymorphism typing described by Hershberg *et al.* [62], the Beijing genotype belongs to the modern lineage, and the EAI genotype seems to be

an evolutionary lineage more closely related to the common ancestor of the *M. tuberculosis* complex.

Another genotype that is more frequent in South East Asia than in other continents is the no-copy genotype (*M. tuberculosis* strains without IS6110 in their genome) [63]. The first no-copy genotype strain was isolated from an Indian patient in 1993 [64]. The prevalence of the no-copy genotype in India (about 11%) was much lower than that of the Beijing genotype [65]. Few studies have observed this genotype elsewhere. Anecdotal evidence shows the no-copy genotype circulates in Vietnam, but up to now no studies have focused on this specific strain. Its frequency in Vietnam is unknown, as are possible associations with drug resistance and other epidemic factors.

Mixed tuberculosis infections

In 1976 there were already anecdotal indications that TB patients can be re-infected by another MTB strain and that infections with multiple strains exist. Using phage typing, Bates *et al*. [66] found different phage types of *M. tuberculosis* in single hosts. After that, TB mixed infections were confirmed by using molecular techniques in populations living in crowded conditions and/or with high HIV prevalence, including a high-density urban community [67] and a hospital [68] in South Africa and a prison in Georgia [69].

However, the frequency of mixed infections in settings with a lower tuberculosis infection pressure (e.g. populations in less crowded conditions) and with lower HIV-prevalence, such as Vietnam, is unknown. Also possible associations between TB mixed infections and poor treatment outcomes or severe symptoms have not been studied.

Drug resistant mutations and treatment outcomes

In Vietnam, isoniazid has been used since 1952 [70]. Since then isoniazid resistance has emerged and risen to relatively high levels with 16.6 % of new TB patients infected with MTB resistant to isoniazid in 2001 [15], and one of the predominant genotypes in Vietnam, the Beijing genotype [55], was also associated with MDR [9], isoniazid and streptomycin resistance [55]. Isoniazid is considered one of the main drugs in the first-line anti-tuberculosis treatment [18]. Isoniazid resistance is often due to mutations in the *katG* 315 codon or in the *inhA* promoter region [21, 23]. Some studies showed that *katG* 315 codon mutations were more frequent among patients infected with Beijing genotype strains [24] and related to MDR [23], furthermore these mutations possibly have higher transmission fitness [27]. These differences in isoniazid resistance-conferring mutations may also be related to other characteristics of MTB strains as well as to treatment outcomes. There are, however, very few studies on the mutations underlying resistance to anti-tuberculosis drugs and treatment outcome. Therefore, the observation of the association between mutations causing isoniazid resistance with genotype as well as with treatment outcomes is important.

VI-Aim of the thesis

This thesis describes the distribution of MTB genotypes, drug resistance mutations and their association with treatment outcomes and other patient factors, as well as assesses the validity of new molecular methods in diagnosis and typing, with the aim of providing an overview of the characteristics of tuberculosis in Vietnam relevant for policies by the Vietnamese National TB Program to improve its management and treatment. Specifically, this thesis attempts to address 5 questions:

1-What is the validity of the Genotype® MTBDR*plus* line-probe assay for detecting multidrug-resistant tuberculosis and what are the frequencies of mutations in the *rpoB* core region, the *katG*315 codon and the *inhA promoter region* in Vietnam?

2- Do mutations in the *katG* 315 codon and in the *inhA promoter region* impact on TB treatment outcomes; and is there any association between those mutations with MTB genotype?

3- Do TB mixed infections and the no-copy IS*6110* strains occur in Vietnam? Are there any associations between these and drug resistance or other epidemic factors?

4- Is VNTR typing truly a good method to replace RFLP typing for discriminating among Beijing genotype strains in Vietnam?

5- Is there any association between MTB genotype and first-line treatment failure or relapse rate in Vietnam?

VII-Outline the thesis

In Chapter 2, we describe the specificity and sensitivity of Genotype® MTBDR*plus* test. We also estimate the frequency of mutations of *rpoB, katG*315 codon and *inhA* promoter region among multidrug-resistant strains collected in a nationwide drug-resistance survey. In Chapter 3, we show that mixed infections were truly present in Vietnam and estimate the proportion of smear-positive TB cases that have mixed infections. We also describe the association between mixed infection with drug resistance and other epidemic factors. In Chapter 4 and 5, we assess the association between genotype and relapse and first-line treatment failure in Vietnam. In Chapter 6, we describe the frequency of mutations in *katG* codon315 and the *inhA* promoter region in strains resistant to isoniazid, and assess the association between those mutations and treatment outcomes and MTB genotype. In Chapter 7, we describe the frequency of no-copy strains in a rural area of Vietnam and the association between these strains and drug resistance as well as other epidemic factors. In Chapter 8, we make a comparison of the discriminating value among Beijing genotype strains of VNTR typing versus that of RFLP typing. Chapter 9 gives an overall discussion of the studies. This is followed by English and Dutch summaries of the thesis.

References

1. Palomino JC, Leão SC, Ritacco V (Eds). Tuberculosis 2007. From basic science to patient care. www.TuberculosisTextbook.com 2007. Available at: http://www.solox2000.com/ pub/tuberculosis2007.pdf
2. Bates JH, Stead WW. The history of tuberculosis as a global epidemic. Med Clin North Am. 1993;77:1205-17.

3. Knechel NA. Tuberculosis: pathophysiology, clinical features, and diagnosis. Crit Care Nurse. 2009;29:34-43; quiz 4.
4. Tiemersma EW, van der Werf MJ, Borgdorff MW, Williams BG, Nagelkerke NJ. Natural history of tuberculosis: duration and fatality of untreated pulmonary tuberculosis in HIV negative patients: a systematic review. PLoS One.6:e17601.
5. World Health Organization. Global Tuberculosis Control. WHO report 2011 Geneva: World Health Organization, 2011.
6. World Health Organization. Global tuberculosis control. WHO report 2000. Geneva: World Health Organization, 2000.
7. World Health Organization. Global Tuberculosis control. WHO report 2012. Geneva: World Health Organization, 2012.
8. Hoa NB, Sy DN, Nhung NV, Tiemersma EW, Borgdorff MW, Cobelens FG. National survey of tuberculosis prevalence in Vietnam. Bull World Health Organ. 2010;88:273-80.
9. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, *et al*. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis. 2009;13:900-6.
10. Espinal MA, Laszlo A, Simonsen L, Boulahbal F, Kim SJ, Reniero A, *et al*. Global trends in resistance to antituberculosis drugs. World Health Organization-International Union against Tuberculosis and Lung Disease Working Group on Anti-Tuberculosis Drug Resistance Surveillance. N Eng J Med. 2001;344(17):1294-303.
11. Crofton J. Possible causes of the failure of the treatment of pulmonary tuberculosis; how to avoid them. Bull Int Union Tuberc. 1980;55:93-101.
12. World Health Organization. The Global Plan To Stop TB 2011-2015. Geneva: World Health Organization, 2011.
13. World Health Organization. Global Tuberculosis Control: Surveillance, planning, financing. WHO report 2007. Geneva: World Health Organization, 2007.
14. Huong NT, Duong BD, Co NV, Quy HT, Tung LB, Bosman M, *et al*. Establishment and development of the National Tuberculosis Control Programme in Vietnam. Int J Tuberc Lung Dis. 2005;9:151-6.
15. Huong NT, Lan NT, Cobelens FG, Duong BD, Co NV, Bosman MC, *et al*. Antituberculosis drug resistance in the south of Vietnam: prevalence and trends. J Infect Dis. 2006;194:1226-32.
16. Quy HT, Lan NT, Borgdorff MW, Grosset J, Linh PD, Tung LB, *et al*. Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate? Int J Tuberc Lung Dis. 2003;7:631-6.
17. World Health Organization. Multidrug and extensively drug-resistant TB (M/XDR-TB). 2010 Global Report On Surveillance and Response. Geneva: World Health Organization, 2010.
18. Wright A, Zignol M, Van Deun A, Falzon D, Gerdes SR, Feldman K, *et al*. Epidemiology of antituberculosis drug resistance 2002-07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance. Lancet. 2009;373:1861-73.
19. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, *et al*. *inhA,* a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis.* Science. 1994;263:227-30.
20. Kelley CL, Rouse DA, Morris SL. Analysis of ahpC gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1997; 41:2057-8.

21. Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D, van Embden JD. Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. J Infect Dis. 1996;173:196-202.
22. Lee AS, Teo AS, Wong SY. Novel mutations in ndh in isoniazid-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother. 2001;45:2157-9.
23. Hillemann D, Kubica T, Agzamova R, Venera B, Rusch-Gerdes S, Niemann S. Rifampicin and isoniazid resistance mutations in *Mycobacterium tuberculosis* strains isolated from patients in Kazakhstan. Int J Tuberc Lung Dis. 2005;9:1161-7.
24. Mokrousov I, Narvskaya O, Otten T, Limeschenko E, Steklova L, Vyshnevskiy B. High prevalence of *KatG* Ser315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996 to 2001. Antimicrob Agents Chemother. 2002;46:1417-24.
25. Piatek AS, Telenti A, Murray MR, El-Hajj H, Jacobs WR, Jr., Kramer FR, *et al*. Genotypic analysis of *Mycobacterium tuberculosis* in two distinct populations using molecular beacons: implications for rapid susceptibility testing. Antimicrob Agents Chemother. 2000;44:103-10.
26. Tracevska T, Jansone I, Broka L, Marga O, Baumanis V. Mutations in the rpoB and *katG* genes leading to drug resistance in *Mycobacterium tuberculosis* in Latvia. J Clin Microbiol. 2002;40:3789-92.
27. Gagneux S, Burgos MV, DeRiemer K, Encisco A, Munoz S, Hopewell PC, *et al*. Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. PLoS Pathog. 2006;2:e61.
28. van Doorn HR, de Haas PE, Kremer K, Vandenbroucke-Grauls CM, Borgdorff MW, van Soolingen D. Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of *katG*: a decade of experience in The Netherlands. Clin Microbiol Infect. 2006;12:769-75.
29. Barnes PF, Cave MD. Molecular epidemiology of tuberculosis. N Eng J Med. 2003;349:1149-56.
30. Bartlett JM, Stirling D. A short history of the polymerase chain reaction. Methods Mol Biol. 2003;226:3-6.
31. Hillemann D, Rusch-Gerdes S, Richter E. Evaluation of the GenoType MTBDR*plus* assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol. 2007;45:2635-40.
32. Van Rie A, Page-Shipp L, Scott L, Sanne I, Stevens W. Xpert((R)) MTB/RIF for point-of-care diagnosis of TB in high-HIV burden, resource-limited countries: hype or hope? Expert Rev Mol Diagn.10:937-46.
33. Hillemann D, Weizenegger M, Kubica T, Richter E, Niemann S. Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2005;43:3699-703.
34. Cavusoglu C, Turhan A, Akinci P, Soyler I. Evaluation of the Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* isolates. J Clin Microbiol. 2006;44:2338-42.
35. Miotto P, Piana F, Penati V, Canducci F, Migliori GB, Cirillo DM. Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strains isolated in Italy. J Clin Microbiol. 2006;44:2485-91.

36. Makinen J, Marttila HJ, MarJAMAki M, Viljanen MK, Soini H. Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 2006;44:350-2.
37. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. J Clin Microbiol. 2006;44:3659-64.
38. Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am J Resp Crit Care Med. 2008;177:787-92.
39. World Health Organization. Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). Policy statement 2008 [cited 4 September 2009]; Available from: http://www.who.int/tb/features_archive/policy_ statement.pdf.
40. van Soolingen D, de Haas PEW, Kremer K. Restriction fragment length polymorphism typing of mycobacteria. In: T. Parish, Stoker NG, editors. In: *Mycobacterium tuberculosis* protocols. Totowa, New Jersey, USA: Humana Press Inc.; 2001. p. 165-203.
41. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, *et al*. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993;31:406-9.
42. Frieden TR, Sherman LF, Maw KL, Fujiwara PI, Crawford JT, Nivin B, *et al*. A multi-institutional outbreak of highly drug-resistant tuberculosis: epidemiology and clinical outcomes. JAMA. 1996;276:1229-35.
43. Daley CL. Tuberculosis recurrence in Africa: true relapse or re-infection? Lancet. 1993;342:756-7.
44. Small PM, McClenny NB, Singh SP, Schoolnik GK, Tompkins LS, Mickelsen PA. Molecular strain typing of *Mycobacterium tuberculosis* to confirm cross-contamination in the mycobacteriology laboratory and modification of procedures to minimize occurrence of false-positive cultures. J Clin Microbiol. 1993;31:1677-82.
45. Braden CR, Templeton GL, Stead WW, Bates JH, Cave MD, Valway SE. Retrospective detection of laboratory cross-contamination of *Mycobacterium tuberculosis* cultures with use of DNA fingerprint analysis. Clin Infect Dis. 1997;24:35-40.
46. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, *et al*. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006;6:23.
47. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, *et al*. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 1997;35:907-14.
48. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, *et al*. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006;44:4498-510.
49. Smittipat N, Billamas P, Palittapongarnpim M, Thong-On A, Temu MM, Thanakijcharoen P, *et al*. Polymorphism of variable-number tandem repeats at multiple loci in *Mycobacterium tuberculosis*. J Clin Microbiol. 2005;43:5034-43.
50. Kremer K, Au BK, Yip PC, Skuce R, Supply P, Kam KM, *et al*. Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates

from Hong Kong and comparison with IS*6110* restriction fragment length polymorphism typing and spoligotyping. J Clin Microbiol. 2005;43:314-20.

51. van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, *et al*. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J Clin Microbiol. 1995;33:3234-8.
52. Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN, *et al*. Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. J Clin Microbiol. 2004;42:4040-9.
53. Glynn JR, Kremer K, Borgdorff MW, Rodriguez MP, Soolingen DV. Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. Emerg Infect Dis. 2006;12:736-43.
54. Filliol I, Driscoll JR, Van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G, *et al*. Global distribution of *Mycobacterium tuberculosis* spoligotypes. Emerg Infect Dis. 2002;8:1347-9.
55. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K, *et al*. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis. 2000;6:302-5.
56. Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerg Infect Dis. 2003;9:1633-5.
57. Sun YJ, Lee AS, Wong SY, Paton NI. Association of *Mycobacterium tuberculosis* Beijing genotype with tuberculosis relapse in Singapore. Epidemiol Infect. 2006;134:329-32.
58. Burman WJ, Bliven EE, Cowan L, Bozeman L, Nahid P, Diem L, *et al*. Relapse associated with active disease caused by Beijing strain of *Mycobacterium tuberculosis.* Emerg Infect Dis. 2009;15:1061-7.
59. Parwati I, Alisjahbana B, Apriani L, Soetikno RD, Ottenhoff TH, van der Zanden AG, *et al*. *Mycobacterium tuberculosis* Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J Infect Dis. 2010;201:553-7.
60. Das S, Paramasivan CN, Lowrie DB, Prabhakar R, Narayanan PR. IS*6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, south India. Tuber Lung Dis. 1995;76:550-4.
61. Park YK, Bai GH, Kim SJ. Restriction fragment length polymorphism analysis of *Mycobacterium tuberculosis* isolated from countries in the western pacific region. J Clin Microbiol. 2000;38:191-7.
62. Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, *et al*. High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. PLoS Biol. 2008;6:e311.
63. Lok KH, Benjamin WH, Jr., Kimerling ME, Pruitt V, Lathan M, Razeq J, *et al*. Molecular differentiation of *Mycobacterium tuberculosis* strains without IS*6110* insertions. Emerg Infect Dis. 2002;8:1310-3.
64. van Soolingen, D, de Haas PE, Hermans PW, Groenen PM, Van Embden JD. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis.* J Clin Microbiol, 1993; 31:1987-95.
65. Chauhan DS, Sharma VD, Parashar D, Chauhan A, Singh D, Singh HB, *et al*. Molecular typing of *Mycobacterium tuberculosis* isolates from different parts of India based on

IS*6110* element polymorphism using RFLP analysis. Indian J Med Res. 2007;125:577-81.

66. Bates JH, Stead WW, Rado TA. Phage type of tubercle bacilli isolated from patients with two or more sites of organ involvement. Am Rev Respir Dis. 1976;114:353-8.
67. Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, Gey van Pittius NC, *et al*. Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Respir Crit Care Med. 2004;169:610-4.
68. Cohen T, Wilson D, Wallengren K, Samuel EY, Murray M. Mixed strain *M. tuberculosis* infections among patients dying in hospital in KwaZulu-Natal, South Africa. J Clin Microbiol. 2010; 49:385-8
69. Shamputa IC, Jugheli L, Sadradze N, Willery E, Portaels F, Supply P, *et al*. Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respir Res. 2006;7:99.
70. Association d'enseignement médical des hôpitaux de Paris. Semains des hospitaux. Paris, 1958;1 as referred to by Dang Vu Xich in: Tập san lao & Bệnh phổi. Report no. 8. Hanoi, Vietnam: Sport and Medicine Publications, 1966.

CHAPTER 2

Validation of the GenoType® MTBDR*plus* assay for diagnosis of multidrug resistant tuberculosis in South Vietnam

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BMC Infectious Diseases 2010, 10:149-156

Abstract

Background
To control multidrug resistant tuberculosis (MDR-TB), the drug susceptibility profile is needed to guide therapy. Classical drug susceptibility testing (DST) may take up to 2 to 4 months. The GenoType® MTBDR*plus* test is a commercially available line-probe assay that rapidly detects *Mycobacterium tuberculosis* (MTB) complex, as well as the most common mutations associated with rifampin and isoniazid resistance.
We assessed sensitivity and specificity of the assay by using a geographically representative set of MTB isolates from the South of Vietnam.

Methods
We re-cultured 111 MTB isolates that were MDR, rifampin-resistant or pan-susceptible according to conventional DST and tested these with the GenoType® MTBDR*plus* test.

Results
By conventional DST, 55 strains were classified as MDR-TB, four strains were rifampicin mono-resistant and 52 strains were susceptible to all first-line drugs. The sensitivity of the GenoType® MTBDR*plus* was 93.1% for rifampicin, 92.6% for isoniazid and 88.9% for the combination of both; its specificity was 100%. The positive predictive value of the GenoType® MTBDR*plus* test for MDR-TB was 100% and the negative predictive value 90.3%.

Conclusions
We found a high specificity and positive predictive value of the GenoType® MTBDR*plus* test for MDR-TB which merits its use in the MDR-TB treatment program in Vietnam.

Background

Tuberculosis (TB) is an important disease in developing countries [1]. A major concern is the occurrence of multidrug resistant (MDR) TB [2], which is characterized by resistance to both rifampicin and isoniazid (INH). MDR-TB is difficult to treat and associated with increased treatment failures and relapses. In patients with previously untreated pulmonary TB, inappropriate treatment may lead to selection for bacteria with drug resistance associated mutations [3].

Vietnam ranks 14th among the countries with the highest burden of TB; the incidence of TB is highest in the southern part of the country [1]. In 2005, South Vietnam notified 29,789 new smear positive cases yielding an estimated prevalence of 92.8 per 100,000 (NTP, unpublished data, 2006). *M. tuberculosis* resistance to INH is common (16-25% among new patients) [4, 5]. Among patients experiencing a first episode of TB these two studies reported MDR-TB rates of 2 and 4%, and of 23% and 27% among previously treated patients, respectively [4,5], whereas 80% chronic patients had MDR-TB [6]. Genetically, approximately half of the strains belongs to the East-African Indian clade whereas the other half are of the Beijing genotype, which was found to be strongly associated with (multi-) drug resistance [7,8].

To control MDR-TB, drug resistance patterns should be available to guide the therapy of the patient. However, phenotypic drug susceptibility testing (DST) is a time-consuming process because it requires culturing, which may take up to two months or longer. As long as no DST results are available, the patient will be treated with standard first-line anti-TB drugs. Rapid diagnosis of MDR-TB will permit an earlier start with second-line drug treatment for patients with MDR-TB and may thus decrease the risk of treatment failure, relapse, amplification of DR, and continuing transmission of MDR-TB.

The vast majority of resistance to rifampicin is caused by mutations located in the 81-bp hotspot region of the *rpoB* gene [9]. Mutations conferring resistance to INH are located at several genomic loci (*katG*, *inhA* and *kasA*) [10-14]. Varying by geographic area, 50 to 100% of INH resistant strains have mutations in codon 315 of the *katG* gene or in the promoter region of the *inhA* gene [13,15,16].

The GenoType® MTBDR*plus* assay (Hain Lifescience, Nehren, Germany) is a commercially available assay that combines detection of MTB complex with prediction of resistance to rifampicin and INH. In the assay a multiplex PCR is followed by hybridization of the obtained DNA amplicons to membrane-bound probes. The assay combines detection of MTB complex with detection of mutations in the 81-bp hotspot region of *rpoB*, at codon 315 of the *katG* gene and in the *inhA* promoter region. It was found to have high sensitivity and high specificity for rifampicin and INH resistance and performs well when applied directly to AFB smear-positive sputum specimens [15, 17-20]. A recent meta-analysis pooled all these studies and calculated pooled sensitivity and specificity rates of 99% (95% confidence interval (CI), 96%-100%) and 99% (95% CI, 98%-100%) respectively for rifampicin resistance, and of 96% (93-98%) and 100% (99-100%), respectively for isoniazid resistance [21].

In June 2008, the World Health Organization (WHO) endorsed the use of molecular line probe assays for MDR-TB screening [22], and the GenoType®

MTBDR*plus* assay has since been introduced for routine practice in various countries [15,17-20,23]. The WHO recommends that before using the assay in routine TB treatment and control, the performance of the assay, in relation to the locally circulating *M. tuberculosis* bacteria, should be validated [22].

The National Tuberculosis Control Program of Vietnam intends to use this test in support of Programmatic Management of DR-TB (PMDT) on sputum specimens of all MDR-TB suspects (i.e., those failing category 1 treatment and those being smear-positive after 3 months of category 2 treatment). The Genotype® MTBDR*plus* assay will be used to select patients with rifampicin resistant isolates for PMDT. Therefore, we assessed the test's sensitivity and specificity in diagnosing MDR-TB at the laboratory of Pham Ngoc Thach Hospital (PNTH) using a geographically representative set of *M. tuberculosis* isolates with known phenotypic resistance patterns from the South of Vietnam.

Methods

Study population

We selected MTB isolates from the latest nationwide TB drug resistance survey (DRS) in Vietnam conducted in 2004-2005. This survey was carefully designed as to cover all geographical parts of Vietnam, and the set of samples thus provided a national estimate of drug resistance for Vietnam. DST was done by the two national reference laboratories, one of which was at PNTH in Ho Chi Minh City. The DRS was part of the WHO/IUATLD Global Project on TB Drug Resistance Surveillance and followed WHO guidelines [24]. PNTH's laboratory participates in an annual international proficiency study on DST together with laboratories in Korea and Australia, with results that are concordant to those of other laboratories for more than 95 % of the tests.

Complete DST results were available for 1,826 patients with smear-positive TB, from 80 different TB clinics throughout the country. Of these, 1,044 (57%) specimens were collected in the South of Vietnam and tested in PNTH (910 from new patients and 134 from previously treated patients). Sputum specimens were processed according to standard procedures [24].

After centrifugation, sediment was inoculated on Löwenstein-Jensen (LJ) medium and incubated at 37°C for 4 to 8 weeks followed by species identification of culture-positive samples. DST for isoniazid (0.2 µg/ml), streptomycin (4 µg/ml), ethambutol (2 µg/ml), and rifampicin (40 µg/ml) was performed using LJ media following the proportion method [25].

Isolates from all 30 new and 29 re-treatment cases that were either identified as MDR-TB (n=55) or resistant to rifampicin (n=4) by phenotypic DST were included in this study. In addition, from the isolates that were susceptible to all tested first-line drugs from new and re-treatment patients, we randomly selected 52 isolates, so that the total number of tested strains was 111.

*GenoType® MTBDR*plus *testing*

GenoType® MTBDR*plus* testing was performed blinded from the phenotypic DST results. The selected MTB isolates were re-cultured from the -70°C freezer and

subjected to the GenoType® MTBDR*plus* test according to the manufacturer's recommendations (www.hainlifescience.de). By multiplex PCR the *rpoB*, *katG* and *inhA* genes were amplified and the resulting biotin-labeled amplicons were hybridized to DNA probes bound to membrane strips. Hybridization was detected by addition of a streptavidin/alkaline phosphatase (AP) conjugate and an AP mediated staining reaction. For each gene, the GenoType® MTBDR*plus* assay tests for presence of so-called wild-type (WT) and mutant (MUT) probes, the first comprising the most important resistant areas of the respective genes and the second some of the most common resistance mediating mutations. Next to that, the TUB zone hybridizes with amplicons generated from all members of the *Mycobacterium* complex and can thus serve for species identification. The membrane-bound DNA probes included eight *rpoB* wild-type probes, four *rpoB* mutant probes (with D516V, H526Y, H526D, and S531L mutations), one *katG* wild-type probe, two *katG* mutant probes (with S315T1 and S315T2 mutations), two *inhA* wild-type probes and four *inhA* mutant probes (with C15T, A16G, T8C, and T8A mutations) [26]. Following the manufacturer's instructions (www.hain-lifescience.de), susceptibility to isoniazid and rifampicin was defined as hybridization to all the wild type probes and no hybridization to the mutant probes. A strain that revealed hybridization to both a mutant probe and to the corresponding wild type probe was considered to represent a heterogeneous population of bacteria or a mixed infection of a sensitive and a resistant strain.

Species identification

M. tuberculosis was identified by smear microscopy followed by a positive niacin reaction [27]. Further species identification was performed using Innolipa Mycobacteria v2 (Innogenetics, Gent, Belgium), if a discrepancy was found between initial species identification and results from the GenoType® MTBDR*plus* assay (i.e., no hybridization with the TUB band).

DNA fingerprinting and sequencing

In order to identify possible cases of mixed infection, spoligotyping [28] and IS*6110* restriction fragment length polymorphism (RFLP) [29] were applied to single colonies growing on standard culture medium and medium supplemented with tuberculostatics. DNA patterns were scanned and analyzed by using Gelcompar software (Applied Maths, Sint-Martens-Latum, Belgium) as previously described [30]. The Beijing genotype was defined by spoligotyping as any isolate without Direct Repeat spacers 1–34 and the presence of ≥3 of the spacers 35–43 [31]. Other genotypes were defined as described by Brudey *et al.* [32]; including the Vietnam genotype (EAI-VNM) that belongs to the East African Indian genotype family of *M. tuberculosis* and is the most frequent genotype in this study site [8].

Sequencing of the rifampin resistance-determining region (RRDR) of the *rpoB* gene was performed for strains that had discordant results for rifampicin according to conventional DST and the GenoType® MTBDR*plus* test. The 350 bp fragment of the *rpoB* gene was amplified using outer primers RPOBF (5′- GGGAGCGGAT GACCACCCA3′) and RPOBR

(5′- CGGTACGGCGTTTCGATGAAC-3′). The primers were designed using Primer Express version 2.0 software (Applied Biosystems Inc, Foster City, CA, USA) [33].

Ethical approval

The study protocol was approved by the Research Board of Pham Ngoc Thach hospital. Since this study used stored isolates with known drug resistance patterns and no additional procedures on the patients were involved; individual informed consent was not obtained.

Statistical methods

Data were double entered in EpiData version 3.1 (http://www.epidata.dk) by two separate study assistants. Discrepancies were checked against the crude data. Chi-squared tests were performed using Epi Info version 6.04 (http://www.cdc.gov/epiinfo/). Results were considered significant at $P < 0.05$.

Results

Concordance between conventional DST and GenoType® MTBDR*plus *assay

Of 111 isolates tested, there was one MDR strain that lacked the TUB band. Although this strain was earlier identified as *M. tuberculosis*, further species identification identified the strain as *M. avium-intracellulare* (MAIS). Spoligotyping after re-culturing this isolate showed that it was a mixture of *M. tuberculosis* and MAIS. Since the GenoType® MTBDR*plus* assay did not identify this strain as MDR-TB, all analyses describing the assay's performance include 110 isolates, of which 58 were resistant to rifampicin by conventional DST.

Based on phenotypic DST, 54 strains were MDR-TB, four strains were rifampicin mono-resistant and 52 strains were susceptible to all first-line drugs. Considering the phenotypic DST method as the gold standard, the GenoType® MTBDR*plus* test correctly identified 48 of 54 MDR-TB strains (88.9%, 95% CI: 77.4-95.8%); 54 of 58 rifampicin resistant strains (93.1%, 95% CI: 83.3-98.1%); 50 of 54 INH resistant strains (92.6%, 95% CI: 82.1-97.9%); and all susceptible strains (100%, 95% CI: 93.2-100 %). The specificity for detecting MDRTB was 100%. The overall concordance of the GenoType® MTBDR*plus* test and phenotypic DST was 94.5% (104/110). Sensitivities, specificities and predictive values are listed in Table 1.

Table 1. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) and 95% confidence intervals of the GenoType® MTBDR*plus* assay on 110 *M. tuberculosis* cultures isolates in the South of Vietnam.

	Rifampicin	Isoniazid	Multi-drug resistance
Sensitivity	93.1% (83.3-98.1)	92.6% (82.1-97.9)	88.9% (77.4-95.8)
Specificity	100% (93.2-100)	100% (93.6-100)	100% (93.6-100)
PPV	100% (93.4-100)	100% (92.9-100)	100% (92.6-100)
NPV	92.9% (82.7-98.0)	93.3% (83.8-98.1)	90.3% (80.1-96.4)

Sequencing the RRDR of the *rpoB* gene of the four strains with discordant results for rifampicin revealed that one strain possessed the H526L mutation which is not present on the membrane strips; in the three remaining strains no mutations were detected in the *rpoB* gene.

Frequency of drug resistance associated mutations

Mutation patterns produced by the GenoType® MTBDR*plus* test are displayed in Table 2. Among the 54 RIF resistant TB strains detected by this test, the frequency of *rpoB* mutations was: 27 S531L (50%), 6 H526Y (10.9 %), 3 D516V (5.5 %), 1 H526D (1.8%), 7 missing WT2 (12.7%), 10 missing WT3 (18.1%), 6 missing WT4 (10.9%), 14 missing WT7 (25.5%), 26 missing WT8 (48%), and no case missing WT1, WT5 or WT6.

Among 50 INH resistant TB strains as identified by the GenoType® MTBDR*plus* test, *katG* mutations occurred in 43 (86%) and *inhA* mutations in 9 strains (18%). Two of the 43 (5%) strains with a *katG* codon 315 mutation had an additional mutation in the *inhA* promoter region. The most frequently observed *katG* mutation was *katG* S315T1 (in 38 of 43 strains, 88.4%), whereas *katG* S315T2 (2.3%) and unknown mutations (i.e., no hybridization to the *katG* WT nor to either of the mutation probes, 9.3%) occurred less frequently. All 9 strains with a mutation in the *inhA* promoter region had an *inhA* C15T mutation (Table 2).

Detection of mixed bacterial populations

With this rapid assay four possible mixtures were detected, although one of these was not identified by the assay as *M. tuberculosis* due to a lacking TUB band and was later identified to contain MAIS. These four phenotypically rifampicin resistant isolates were demonstrated to carry mutations in the *rpoB* gene and/or in the *katG* gene or the *inhA* promoter region, but did not lack hybridization on any of the wild type probes. By using DNA fingerprinting one of these isolates was confirmed to be a mixture of two MTB strains (spoligotype T1 and an undefined type; RFLP type T1 and Beijing), and the isolate lacking the TUB band (number 12647) was identified as a mixture of a MTB strain (spoligotype U) and a non-tuberculous *mycobacterium* (note that spoligotyping yielded a weak signal for *M. tuberculosis* for one single colony culture of this isolate) (Table 3, Figure 1). In the two remaining samples mixed bacterial populations could not be detected. After spoligotyping which revealed no differences as it has a very low resolution among Beijing strains, we also performed IS*6110* RFLP typing on single colony cultures of each of these two samples and found they all had identical banding patterns (Table 3).

Discussion

In this study, the GenoType® MTBDR*plus* assay correctly identified 93.1%, 92.6% and 88.9% of the rifampicin, INH and combined resistance (MDR), respectively and had 100% specificity for each. The sensitivity of detection of rifampicin resistance was similar to that reported from Germany, Italy, Finland, France, Denmark, Turkey and Taiwan (92-100%, $p>0.05$) [15,17-20,26,34,35]. The GenoType® MTBDR*plus*

assay failed to detect four (6.8%) of the rifampicin resistant strains in our study, which was caused either by a rare mutation which is not present on the strips (n=1) or by probable mutations in other genomic regions of the *rpoB* gene (n=3). The sensitivity for detection of isoniazid resistance in our study was 91.2%, which was similar to reports from Germany, Finland, Denmark and Taiwan (84 –100%, p>0.05) [15,17-20,26,34,35], but higher than reported from Turkey, Italy, France and the Caribbean (35-73%, P<0.05) [17,18,20,36]. For MDR-TB the sensitivity of the test was somewhat lower than reported from other research [21].

Table 2. Mutation patterns following from the GenoType ® MTBDR*plus* assay.

rpoB mutations	*katG* mutations	*inhA* mutations	Frequency	Proportion
D516V	S315T1	--	1	0.9
D516V	unknown *	--	1	0.9
D516V	--	C15T	1	0.9
H526D	S315T1	--	1	0.9
H526Y	S315T1	--	5	4.6
S531L	unknown *	--	2	1.8
S531L	S315T1	C15T	1	0.9
S531L	S315T1	--	16	14.6
S531L	S315T2	--	1	0.9
S531L	--	C15T	4	3.6
S531L	--	--	2	1.8
H526Y + S531L	--	--	1	0.9
unknown *	S315T1	--	13	11.8
unknown *	--	--	3	1.8
unknown *	unknown *	C15T	1	0.9
unknown *	--	C15T	1	0.9
--	S315T1	--	1	0.9
--	--	C15T	1	0.9
Total number of strains with any mutations			**56**	**50.9**
--	--	--	54	49.1
Total number of strains			**110**	**100**

* unknown mutation: no hybridization to one or more of the wild type probes nor to any of the mutation probes.

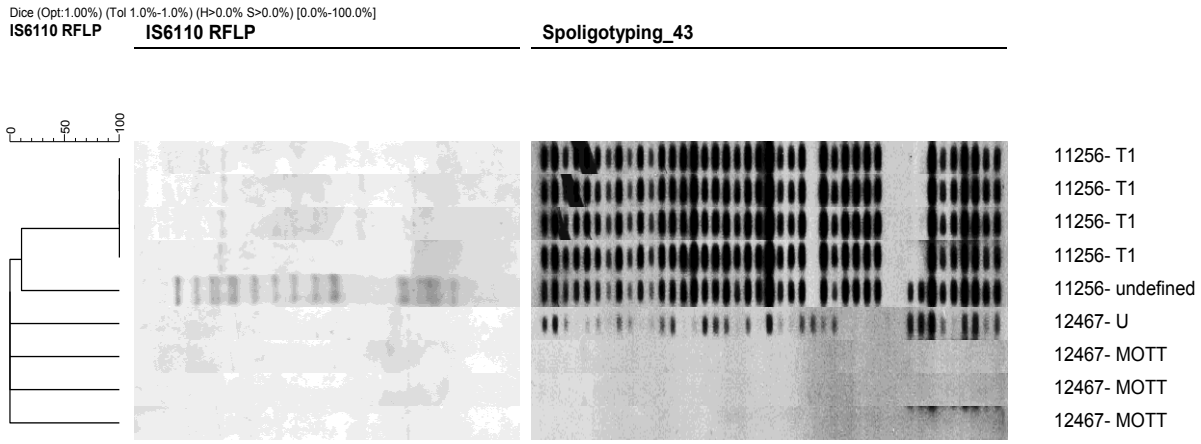

Figure 1. Patterns of multiple *Mycobacterium spp.* Infections as obtained by RFLP and spoligotyping techniques. The first four isolates have only one band by RFLP (non-Beijing) and spoligotype T1; the 5th sample is a mixture of Beijing genotype (as obtained by RFLP) and spoligotype T1. The 6th sample has no RFLP banding pattern and an undefined spoligotype, whereas for the last three isolates no banding patterns were found by RFLP and spoligotyping, probably as a result of the presence of *non-tuberculous mycobacteria* (NTM or MOTT).

The distribution of mutations identified in our study is significantly different from that reported from other continents. We found that the S531L mutation in the *rpoB* gene occurred most frequently (50%) among rifampicin resistant strains. This mutation occurred even more frequently in a collection of isolates from South Africa (70.5%, p=0.014) [23]. In our study only 36 (66.7%) MDR-TB strains had a mutation that could be identified by one of the four *rpoB* mutant probes present on the strip, whereas this was much higher in South Africa (91% (81/89)) [23]. Thus, the assay performs better detecting *rpoB* specific mutations that confer rifampicin resistance in South Africa than in Vietnam. For INH resistance, mutations in the *katG* gene were by far most common (86%) and the S315T1 mutation was found most frequently (88.4%) in our population. From South Africa, Van Rie *et al*. [37] reported similar results, whereas Barnard and colleagues [23] reported that this mutation occurred less frequently (37.6%; p=0.01). In our study 18% of INH resistant MTB strains carried a mutation in the *inhA* promoter region (invariably C15T) which is considerably lower than the 40% reported in Barnard's study (p= 0.007). It should be noted that we only tested MDR-TB isolates, whereas Barnard's study also included INH mono-resistant strains and MDR-TB may be primarily associated with *katG* mutations that generally confer high levels of INH resistance [38].

The GenoType® MTBDR*plus* assay can detect mixtures of drug resistant and drug susceptible bacterial populations. In this study four mixtures were found: three mixtures of *M.tuberculosis* strains and one mixture of a *M. tuberculosis* strain and an *M. avium* complex strain. This indicates that even in a high prevalence area like Vietnam a minor proportion of the TB cases is caused by a mixture of *non-tuberculous*

mycobacteria and MTB. The GenoType® MTBDR*plus* assay may not be sensitive enough to be used for species identification in case of mixed bacterial populations, since the *M. tuberculosis* and *M. avium* mixture revealed no TUB band.

Mixed infections were confirmed by typing a limited number of single colony cultures by spoligotyping and RFLP. In two samples that seemed to consist of a mixture when tested with the GenoType® MTBDR*plus* assay, presence of a mixed infection could not be confirmed which could have been the result of testing only a limited number of single colony cultures. With the 100% specificity of the GenoType® MTBDR*plus* assay to detect MDR in *M. tuberculosis* isolates, no patient would be inappropriately treated with category 4 (MDR-TB) treatment if this test would be used in routine for rapid MDR-TB diagnosis. On the other hand, 6.9% of patients would not receive appropriate category 4 treatment (which is based on detection of rifampicin resistance) if identification of MDR-TB patients is done using only the GenoType® MTBDR*plus* test.

Conclusions

Overall, the GenoType® MTBDR*plus* test is reliable, rapid and easy to perform for the simultaneous detection of rifampicin and INH resistance in *M. tuberculosis*. With high sensitivity for detection of rifampin resistance and high specificity for MDR, we conclude that this test strongly facilitates adequate treatment of MDR-TB patients, long before the results of conventional DST are available. Because discordance still exists between the conventional and molecular approach of DST and susceptibility of bacteria to drugs is defined as inhibition of growth, we recommend that the GenoType® MTBDR*plus* test should serve as an early guidance of therapy, which should be followed by a phenotypic DST confirmation for all suspected MDR-TB patients. Incorporation of the molecular test in the National Tuberculosis Program is an important step forward in the rapid diagnosis of MDR-TB among suspected patients in the PMDT program. The application of the molecular test directly to clinical material with sufficient bacteria will further speed up the turnaround time of the rapid diagnosis of MDR-TB and will be the next step of implementation.

Acknowledgements

This research was funded by the Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland, and the KNCV Tuberculosis Foundation, The Hague, The Netherlands.

We thank the staff of the laboratory of Pham Ngoc Thach Hospital for performing DST and GenoType® MTBDR*plus* testing and for patient recruitment.

We thank the staff of the *Mycobacterium* laboratory of the Oxford University Clinical Research Unit for sequencing and identification of non-tuberculous mycobacteria.

We thank Yuli You at Biomedia Group, Singapore, and staff at the Tuberculosis Reference Laboratory, RIVM, Bilthoven, The Netherlands for providing technical assistance.

Table 3. Identification of mixed TB infection by spoligotyping and RFLP.*

Strain no.	TUB	Rifampin		Isoniazid		Spoligotype	RFLP type + Spoligotype
		WT	MUT	WT	MUT		
10484	+	+	Mut 2A & 3	+	-	Beijing	Identical Beijing
11256	+	+	Mut3	+	*katG* Mut1 & *inhA* Mut1	T1+ undefined type	T1 + Beijing
11901	+	+	Mut3	+	*inhA* Mut1	Beijing	Identical Beijing
12467	-	-	+	+	*katG* Mut1	U + No band	U + No band

* TUB: *M. tuberculosis* confirmation band; WT: wild type; MUT: mutant

References

1. WHO (2009) Global tuberculosis control: surveillance, planning, financing. WHO report 2009. Geneva: World Health Organization. WHO/HTM/TB/2009.411. 294 p.
2. Wright A, Zignol M, Van Deun A, Falzon D, Gerdes SR, *et al*. (2009) Epidemiology of antituberculosis drug resistance 2002-07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance. Lancet 373: 1861-73.
3. Mitchison DA (1998) How drug resistance emerges as a result of poor compliance during short course chemotherapy for tuberculosis. Int J Tuberc Lung Dis 2: 10-5.
4. Quy HT, Buu TN, Cobelens FG, Lan NT, Lambregts CS, *et al*. (2006) Drug resistance among smear-positive tuberculosis patients in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 10: 160-6.
5. Huong NT, Lan NT, Cobelens FG, Duong BD, Co NV, *et al*. (2006) Antituberculosis drug resistance in the south of Vietnam: prevalence and trends. J Infect Dis 194: 1226-32.
6. Quy HT, Lan NT, Borgdorff MW, Grosset J, Linh PD, *et al*. (2003) Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate? Int J Tuberc Lung Dis 7: 631-6.
7. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, *et al*. (2000) *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 6: 302-5.
8. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, *et al*. (2009) The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis 13: 900-6.
9. Telenti A, Imboden P, Marchesi F, Lowrie D, Cole S, *et al*. (1993) Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. Lancet 341: 647-50.
10. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, *et al*. (1994) *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 263: 227-30.
11. Kelley CL, Rouse DA, Morris SL (1997) Analysis of ahpC gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 41: 2057-8.
12. Mdluli K, Slayden RA, Zhu Y, Ramaswamy S, Pan X, *et al*. (1998) Inhibition of a *Mycobacterium tuberculosis* beta-ketoacyl ACP synthase by isoniazid. Science 280: 1607-10.
13. Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D, *et al*. (1996) Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. J Infect Dis 173: 196-202.
14. Ramaswamy S, Musser JM (1998) Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis 79: 3-29.
15. Hillemann D, Weizenegger M, Kubica T, Richter E, Niemann S (2005) Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol 43: 3699-703.

16. Hillemann D, Kubica T, Agzamova R, Venera B, Rusch-Gerdes S, *et al*. (2005) Rifampicin and isoniazid resistance mutations in *Mycobacterium tuberculosis* strains isolated from patients in Kazakhstan. Int J Tuberc Lung Dis 9: 1161-7.

17. Cavusoglu C, Turhan A, Akinci P, Soyler I (2006) Evaluation of the Genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* isolates. J Clin Microbiol 44: 2338-42.

18. Miotto P, Piana F, Penati V, Canducci F, Migliori GB, *et al*. (2006) Use of genotype MTBDR assay for molecular detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* clinical strains isolated in Italy. J Clin Microbiol 44: 2485-91.

19. Makinen J, Marttila HJ, MarJAMAki M, Viljanen MK, Soini H (2006) Comparison of two commercially available DNA line probe assays for detection of multidrug-resistant *Mycobacterium tuberculosis*. J Clin Microbiol 44: 350-2.

20. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W (2006) Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. J Clin Microbiol 44: 3659-64.

21. Bwanga F, Hoffner S, Haile M, Joloba ML (2009) Direct susceptibility testing for multi drug resistant tuberculosis: a meta-analysis. BMC Infect Dis 9: 67.

22. WHO (2008) Molecular line probe assays for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). Policy statement. Geneva: World Health Organization.

23. Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME (2008) Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am J Respir Crit Care Med 177: 787-792.

24. WHO (2003) Guidelines for the surveillance of drug resistance in tuberculosis. Geneva: World Health Organization. WHO/CDS/TB/2003.320. 1-21 p.

25. Canetti G, Rist N, Grosset J (1963) Mésure de la sensibilité du bacilli tuberculeux aux drogues antibacilares par la méthode des proportions. Rev Tuberc Pneumol (Paris) 27: 217-72.

26. Vijdea R, Stegger M, Sosnovskaja A, Andersen AB, Thomsen VO, *et al*. (2008) Multidrug-resistant tuberculosis: rapid detection of resistance to rifampin and high or low levels of isoniazid in clinical specimens and isolates. Eur J Clin Microbiol Infect Dis 27: 1079-86.

27. Kent PT, Kubica GP (1985) Public Health Mycobacteriology. A Guide for Level III Laboratory. In: U.S. Department of Health and Human Services, editor. Atlanta, GA Centers for Disease Control and Prevention. pp. p94-96.

28. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, *et al*. (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35: 907-14.

29. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, *et al*. (1993) Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommen-dations for a standardized methodology. J Clin Microbiol 31: 406-9.

30. Heersma HF, Kremer K, van Embden JD (1998) Computer analysis of IS*6110* RFLP patterns of *Mycobacterium tuberculosis*. Methods Mol Biol 101: 395-422.

31. Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, *et al*. (2004) Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. J Clin Microbiol 42: 4040-9.

32. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, *et al*. (2006) *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 6: 23.

33. Stewart JE, Aagaard PJ, Pokorak EG, Polanskey D, Budowle B (2003) Evaluation of a multicapillary electrophoresis instrument for mitochondrial DNA typing. J Forensic Sci 48: 571-80.

34. Hillemann D, Rusch-Gerdes S, Richter E (2007) Evaluation of the GenoType MTBDR*plus* assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol 45: 2635-40.

35. Huang WL, Chen HY, Kuo YM, Jou R (2009) Performance assessment of the GenoType MTBDR*plus* test and DNA sequencing in detection of multidrug-resistant *Mycobacterium tuberculosis*. J Clin Microbiol 47: 2520-4.

36. Akpaka PE, Baboolal S, Clarke D, Francis L, Rastogi N (2008) Evaluation of methods for rapid detection of resistance to isoniazid and rifampin in *Mycobacterium tuberculosis* isolates collected in the Caribbean. J Clin Microbiol 46: 3426-8.

37. Van Rie A, Warren R, Mshanga I, Jordaan AM, van der Spuy GD, *et al*. (2001) Analysis for a limited number of gene codons can predict drug resistance of *Mycobacterium tuberculosis* in a high-incidence community. J Clin Microbiol 39: 636-41.

38. van Doorn HR, de Haas PE, Kremer K, Vandenbroucke-Grauls CM, Borgdorff MW, *et al*. (2006) Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of *katG*: a decade of experience in The Netherlands. Clin Microbiol Infect 12: 769-75.

CHAPTER 3

Mixed tuberculosis infections in rural South Vietnam

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Journal of Clinical Microbiology 2012, 50(5):1586-1592

Abstract

Background

Tuberculosis patients may be infected with or have disease caused by more than one *Mycobacterium tuberculosis* strain, usually referred to as "mixed infections." These have mainly been observed in settings with a very high tuberculosis incidence and/or high HIV prevalence. We assessed the rate of mixed infections in a population-based study in rural Vietnam, where the prevalences of both HIV and tuberculosis are substantially lower than those in previous studies looking at mixed infections.

Methods

In total, 1,248 *M. tuberculosis* isolates from the same number of patients were subjected to IS*6110* restriction fragment length polymorphism (RFLP) typing, spoligotyping, and variable-number-tandem-repeat (VNTR) typing. We compared mixed infections identified by the presence of (i) discrepant RFLP and spoligotype patterns in isolates from the same patient and (ii) double alleles at ≥ 2 loci by VNTR typing and assessed epidemiological characteristics of these infections.

Results

RFLP/spoligotyping and VNTR typing identified 39 (3.1%) and 60 (4.8%) mixed infections, respectively (Cohen's kappa statistic, 0.57). The number of loci with double alleles in the VNTR pattern was strongly associated with the proportion of isolates with mixed infections according to RFLP/ spoligotyping (P<0.001). Mixed infections occurred more frequently in newly treated than in previously treated patients, were significantly associated with minor X-ray abnormalities, and were almost significantly associated with lower sputum smear grades.

Conclusions

Although the infection pressure in our study area is lower than that in previously studied populations, mixed *M. tuberculosis* infections do occur in rural South Vietnam in at least 3.1% of cases.

Introduction

For a long time it was assumed that a tuberculosis (TB) infection protects against a subsequent infection. In fact, vaccination against infectious diseases is based on this principle. However, in 1976 there were already anecdotal indications that TB patients can be re-infected by another *Mycobacterium tuberculosis* strain and that infections with multiple strains exist. Using phage typing, Bates *et al*. [2] found different phage types of *M. tuberculosis* in single hosts. The occurrence of infections with multiple *M. tuberculosis* strains was confirmed by using DNA fingerprinting techniques, first at the turn of the century in selected patients by Yeh *et al*. [25] and by Braden *et al*. [3] and then more recently in larger patient populations [14, 15, 22]. The introduction of molecular techniques offered new possibilities for studying the natural history of TB infection more extensively. PCR assays targeting particular predominant *M. tuberculosis* genotype families were developed, and by applying these methods to clinical material, Warren *et al*. found that the rate of occurrence of "mixed infections," i.e., infections with multiple *M. tuberculosis* strains, amounted to 19% of examined patients in South Africa [22].

High rates of mixed infections have been found in populations living in crowded conditions, including a high-density urban community [22] and a hospital [7] in South Africa and a prison in Georgia [15]. However, the frequency of mixed infections in human populations with a lower tuberculosis infection pressure (e.g., populations under less crowded conditions and with a lower HIV prevalence) is unknown. Although the burden of TB is high in South Vietnam, with a prevalence of smear-positive TB of 219/100,000 population (95% confidence interval [CI], 145 to 294/100,000) (9), it is much lower than the prevalence in the studied areas in South Africa (1,000/100,000) [22]. Similarly, the prevalence of HIV among TB patients is lower in Vietnam than in South Africa (8.2% versus 50 to 80%) [18; Brand South Africa Media Service].

We studied the occurrence of mixed infections in a population-based study in a rural area in South Vietnam using IS*6110* restriction fragment length polymorphism (RFLP) typing and spoligotyping [11, 20]. The former method is used to distinguish *M. tuberculosis* isolates at the strain level to study patient-to-patient transmission but also enables determination of the genotype to which the *M. tuberculosis* strain belongs, while the latter method can be used only for genotype determination [4]. It is known from previous studies that about 35% of the *M. tuberculosis* isolates from South Vietnam are of the Beijing genotype, while about 49% of the isolates represent the East African Indian (EAI) genotype (previously known as the Vietnam genotype) [1, 5]. The predominance of these two *M. tuberculosis* genotype families in South Vietnam and their highly characteristic IS*6110* RFLP and spoligotype patterns enabled us to detect possible mixed infections with strains of these different genotypes in a reliable way by comparing the results of both typing methods. In addition, mixed infections were detected by the visualization of double alleles in variable number-tandem-repeat (VNTR) typing patterns [13, 15]. In this study, we were able to compare the sensitivity of the various typing approaches to detect mixed infections; however, because much is unknown about the evolution of VNTR patterns, we used RFLP/spoligotype results to quantify the occurrence of mixed infections and investigate possible risk factors for

mixed infections. This is the first report of mixed infections in rural Vietnam, where the population density and HIV infection prevalence are low and TB incidence is moderate, in contrast to settings in previous studies looking at mixed infections.

Materials and methods

Patient population

The study area consisted of three adjacent rural districts in Tien Giang Province, situated in the Mekong River Delta in southern Vietnam. All patients aged ≥15 years, resident in the study area, and registered for treatment of smear-positive pulmonary TB between 1 January 2003 and 31 December 2005 at the participating District Tuberculosis Units or at the provincial TB hospital were eligible for inclusion in the study [10]. By interviews using pre-structured questionnaires, we collected data on the sex, age, *M. bovis* BCG vaccination, X-ray abnormalities, educational level, marital status, occupation, and history of treatment of all participants. HIV testing was not done routinely. Treatment outcomes were based on routine smear examination [24], as described by Buu *et al*. [5]. Buu *et al*. previously found that tuberculosis is usually transmitted outside the household in the study area [6]; therefore, epidemiological links between patients were not assessed in in-depth interviews for the purpose of this study.

Mycobacterial isolates

Sputum specimens were kept refrigerated and were transported to Pham Ngoc Thach Tuberculosis and Lung Disease Hospital in Ho Chi Minh City, Vietnam, within 72 h after collection. Specimens were decontaminated and liquefied with 1% *N*-acetylcysteine, 2% NaOH, inoculated on modified Ogawa medium, and incubated at 37°C. Cultures were examined for growth after 1, 2, 4, 6, and 8 weeks of incubation. Cultures with no growth after 8 weeks were reported to be negative. *M. tuberculosis* was identified using the niacin and nitrate tests [10].

Drug susceptibility testing

Drug susceptibility testing was performed by the proportion method following the guidelines of the World Health Organization and the International Union against Tuberculosis and Lung Disease [23]. Criteria for drug resistance were ≥ 1% colony growth [10] at 28 or 40 days compared to the growth on the drug-free control medium at the following drug concentrations: isoniazid, 0.2 μg/ml; rifampin, 40 μg/ml; streptomycin, 4 μg/ml; and ethambutol, 2 μg/ml. Multidrug resistance (MDR) was defined as resistance to both rifampin and isoniazid.

DNA typing

We included all 1,248 patients (66% of the eligible population) with isolates on which all 3 typing methods were applied (RFLP, spoligo-, and VNTR typing). Genomic DNA was extracted from positive cultures by using a method described earlier [21]. IS*6110* RFLP typing and spoligotyping were performed according to the internationally standardized methods [11, 20]. VNTR typing was done using 15 loci, as described by Supply *et al*. [17].

Definition of mixed infections by RFLP, spoligo and VNTR typing

All isolates that yielded discrepant results with regard to *M. tuberculosis* genotype family in RFLP and spoligotyping were subjected to both typing methods for a second time from the same DNA to ensure the reproducibility of the observation. Beijing and EAI genotypes were assigned as reported elsewhere on the basis of the IS*6110* RFLP and spoligotyping patterns [1, 4, 12]. Isolates that repeatedly had a spoligotype characteristic of the EAI genotype and an IS*6110* RFLP pattern characteristic of a Beijing genotype strain [12], or the other way around, or a spoligotype characteristic of the Beijing or EAI genotype and an IS*6110* RFLP pattern characteristic of another genotype, or the other way around, were considered to represent a mixed infection. VNTR typing has been shown to be sensitive in the detection of mixed infections by revealing double alleles, with mixed infections defined as double alleles in two or more VNTR loci [13, 15]. Because mixtures of two strains of the same genotype are virtually impossible to detect by using only RFLP or spoligotyping, we used the VNTR typing results to check the number of potentially missed mixed infections. We defined the occurrence of double alleles at at least two VNTR loci to be potential mixed infections. However, at present, the sensitivity of VNTR typing for the detection of mixed infections is unknown and double alleles could also represent evolution of the bacterium. Therefore, we do not report the mixed-infection rate according to VNTR typing, but we typed all 1,248 isolates by VNTR, RFLP, and poligotyping to compare their sensitivities for the detection of mixed *M. tuberculosis* infections as a first step for future studies. Furthermore, VNTR typing was done since the data were also collected for other studies performed to present an overview of genotypes in this part of Vietnam and to check for relapse versus new infections.

Re-culture of sputum for analysis of single colonies

We repeated RFLP and spoligotyping twice for all 39 mixed infections from their DNA (extracted from cultures), and we got the same results. Ideally, to exclude mixed infections that occur due to cross-contamination during the culture processing, studies on mixed infections should be performed on original sputum samples. For two of the assumed mixed-infection isolates, we tried to confirm the observation and exclude laboratory cross-contamination by using a bacteriological approach. Pretreated sputum specimens, stored at -20°C, were recultured on 7H10 agar plates to grow single colonies. Spoligotyping was applied to five or six single colonies of each of the isolates.

Data analysis

The Gene Marker software, version 1.5 (Softgenetics, PA), was used for analysis and automated allele calling of the VNTR patterns. The Bionumerics software, version 3.0 (Applied Maths, Sint-Martens Latem, Belgium), was used for the analysis and comparison of IS*6110* RFLP, spoligotype, and VNTR patterns. Data were entered into Epi Info software, version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA). Double entry was done on a 20% random sample (50% in 2003) of all records. Discrepancies were observed in <1% of all records and in <0.05% of all fields. Analyses were performed in Epi Info, version 6.04, and Stata, version 10SE (Stata Corporation, College Station, TX).

For comparison of categorical variables, we used the chi-square and two-sided Fisher's exact tests as appropriate, with trends across ordered categories assessed by Cuzick's test for trend. Results were considered significant at *P* values of <0.05. Associations between mixed infections and explanatory variables were expressed as odds ratios; confounding effects were investigated by stratified analysis using the Mantel-Haenszel test.

Results

During the period from January 2003 to December 2005, 1,890 patients were eligible for inclusion, and isolates from 1,248 (66%) of those patients had complete IS*6110* RFLP, spoligo-, and VNTR typing results and were available for the analyses. Of these, 931 (74.6%) isolates were from male patients and 317 (25.4%) were from female patients with a median age of 50 years (25^{th} and 75^{th} percentiles, 37 and 66 years, respectively). Eleven hundred seven (88.7%) were new patients, 139 (11.1%) were relapse patients, and the remaining 2 cases were of unknown status.

Identification of mixed infections identified by IS6110 RFLP, spoligotyping and VNTR typing

Thirty-nine (3.1%) of 1,248 isolates had RFLP and spoligotype patterns that represented different *M. tuberculosis* genotype families and were considered to be mixed infections. Repeated RFLP and spoligotype analysis from the same DNA confirmed the mixed infections for all 39 isolates. Twenty-eight isolates (71.8%) represented a mixture of Beijing and EAI strains, 5 isolates (12.8%) were a mixture of a Beijing strain and a strain of another genotype (not EAI), 5 isolates (12.8%) were a mixture of an EAI strain and a strain of another genotype (containing more than 4 IS*6110* copies in RFLP analysis and not belonging to the Beijing genotype), and 1 (2.6%) was a mixture of a Haarlem and another strain (Figure 1). Overall, the study population, including the mixed-infection isolates, contained 549 (42.7%) EAI strains, 461 (35.8%) Beijing strains, and 277 (21.5%) strains of other genotypes. These rates of EAI and Beijing genotypes of *M. tuberculosis* were similar to those reported previously for the prevalence of these genotypes in rural areas of South Vietnam (49% for EAI and 35% for Beijing [5]), but the prevalence of the Beijing genotype was lower than that observed in Hanoi and Ho Chi Minh City [1].

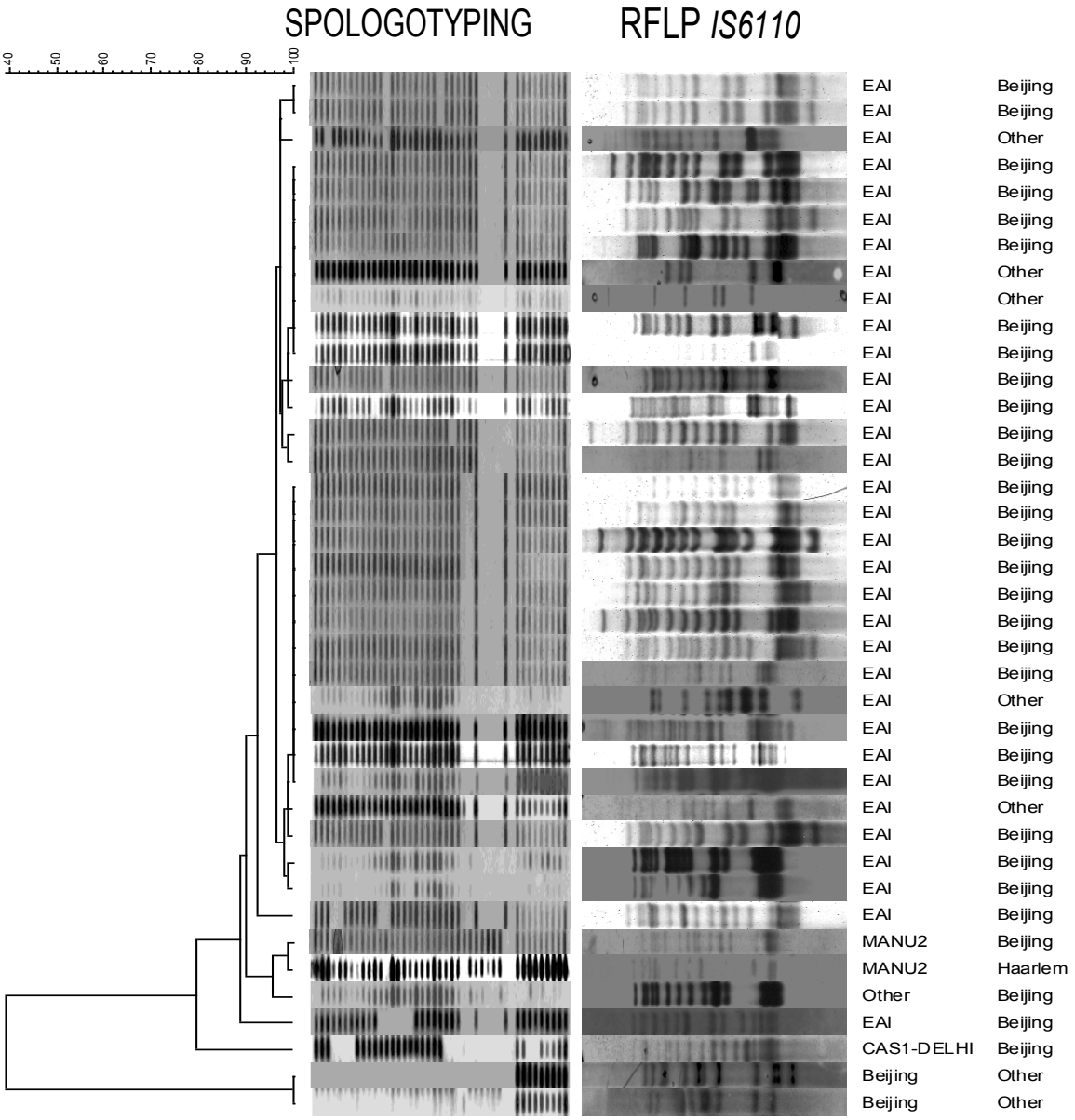

Figure 1. Mixed infections detected by IS*6110* RFLP and spoligotyping. Spoligotypes and IS*6110* RFLP patterns of the *M. tuberculosis* isolates that were identified as mixed infections on the basis of discordant genotyping results. The dendrogram (shown on the left) shows the similarity of the spoligotype patterns, as determined by using the Dice coefficient and unweighted-pair group method using average linkages for clustering. To the right of the IS*6110* RFLP patterns, the interpreted genotype families are indicated, as determined by spoligotyping and IS*6110* RFLP typing, respectively.

VNTR typing detected 122 (9.8%) of 1,248 isolates with double alleles at at least one locus, and 60 (4.8%) of these isolates revealed double alleles at two or more loci (Table 1). Of these 60 isolates, 31 (51.7%) isolates had not been identified as being mixed infections by combining the results of RFLP and spoligotyping and 29 (48.3%) isolates represented mixed infections confirmed by RFLP and spoligotyping. Thus, of the 39 mixed-infection isolates detected by RFLP and spoligotyping, 29 (74.4%) were confirmed by VNTR typing (Table 1). Comparing the RFLP/ spoligotyping and VNTR typing results for the isolates revealed that the percentage of mixed infections detected by RFLP and spoligotyping of isolates strongly increased with the number of loci at which double alleles were found ($P<0.001$; Figure 2). While only 3.2% (2/62) of isolates contained mixed infections (on the basis of RFLP/spoligotyping), when only a single VNTR locus had double alleles, this proportion was more than 80% for isolates that had double alleles in five or more VNTR loci. Among isolates that had two, three, or four loci with double alleles, the proportions of mixed infections were 12.5%, 20.0%, and 44.4%, respectively. Of 1,126 strains having single alleles, 0.7% (8/1,126) were mixed infections, according to combined RFLP and spoligotyping analysis (Table 1). The agreement between the two definitions that we used in this study (discrepant genotype results between RFLP and spoligotyping and double alleles at at least 2 loci with VNTR typing) was 96.7% (Table 1), and Cohen's kappa statistic was 0.57.

Of the 60 isolates that had two or more double alleles in VNTR typing, 29 represented a mixed infection on the basis of the combination of the RFLP and spoligotype results; 25 of these were a mixture of Beijing and EAI genotypes, 3 were a mixture of EAI and another genotype, and 1 was a mixture of a Beijing and another genotype. For 31 of the 60 isolates that had two or more double alleles in VNTR typing, no mixed infection was detected with RFLP and spoligotyping.

Table 1. Comparison of sensitivity of detection of mixed *M. tuberculosis* infections by VNTR typing and by combined typing with IS*6110* RFLP and spoligotyping

Pattern by IS*6110* RFLP and spoligotyping combined	No. of isolates showing the following by 15-locus VNTR typing			Total
	Double alleles in two or more loci	Double allele at one locus	All single alleles	
Mixed infection				
Yes	29	2	8	**39**
No	31	60	1118	1209
Total	**60**	62	1126	1248

Re-culturing of mixed infection isolates

Since identification of mixed infection by culture was not the primary purpose of this study, no attention was given to the phenotypic nature of the cultures. Only after we discovered several samples to contain mixed infections did we decide to reculture these.

Unfortunately, enough material to reculture the sputum sample was available for only two samples. For two of the assumed mixed infections for which original sputum specimens were still available, pretreated sputum specimens were recultured directly on 7H10 agar plates and spoligotyping was applied to five or six single colonies of each of these two isolates. For one specimen, three colonies yielded Beijing-specific spoligotype patterns, while two other colonies revealed an EAI spoligotype. For the other specimen, only Beijing spoligotypes were obtained. Thus, a mixed infection was confirmed microbiologically for one of the two isolates.

In terms of microbiology, we observed that the colonies of Beijing genotype isolates were often smooth and large, with a diameter of more than 2 mm, while the colonies of EAI genotype strains were often dry and small, with a diameter of less than 1 mm.

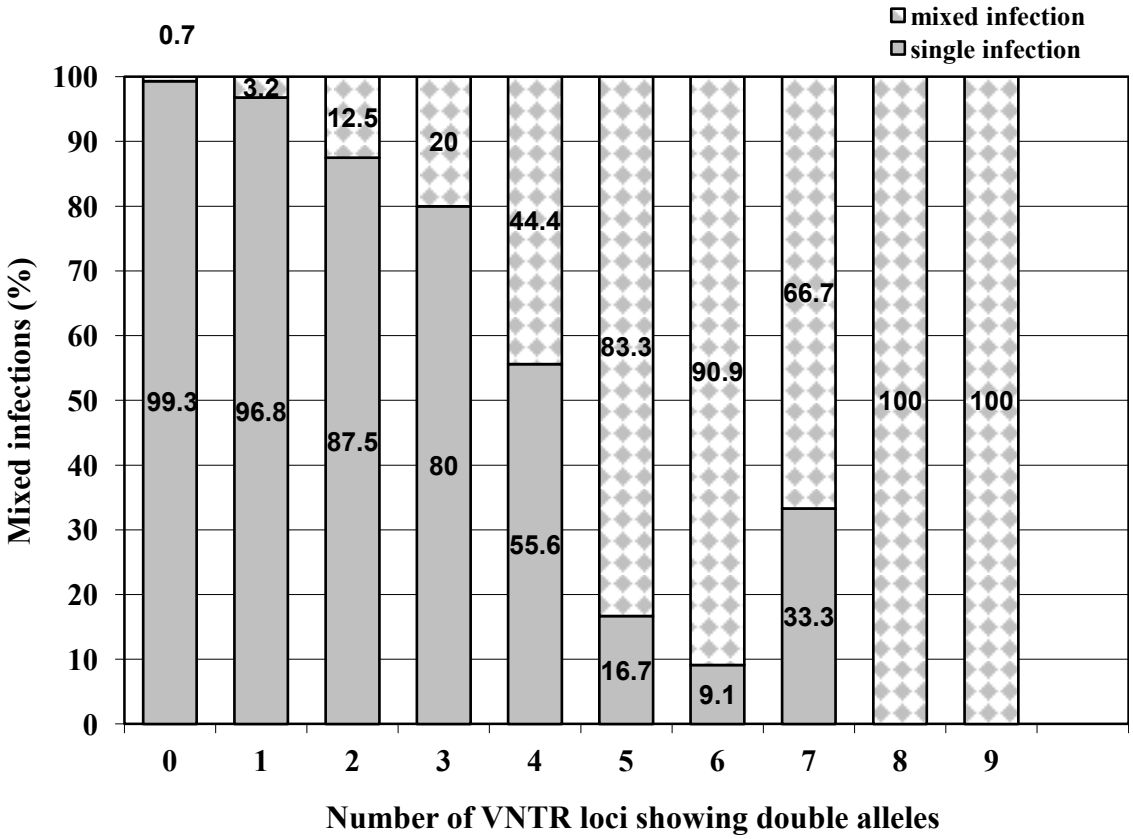

Figure 2. Correlation between the heterogeneity in VNTR patterns and the detection of mixed infections by combined IS*6110* RFLP and spoligotyping among 1,248 *M. tuberculosis* isolates. The percentage of mixed infections by (IS*6110* RFLP/spoligotyping) is indicated for isolates showing VNTR patterns with no double alleles (n = 1,126), a double allele at 1 VNTR locus (n =62), or double alleles at 2 to 9 different VNTR loci (n = 60).

Table 2. Patient and TB disease characteristics for patients with mixed *M. tuberculosis* infections.

Characteristic	No. (%) of patients		P value[a]
	Total	Mixed infections	
Total	1248	39 (3.1)	
District			
Cai Lay	575	22 (3.8)	0.299
Chau Thanh	392	8 (2.0)	
Cai Be	281	9 (3.2)	
Sex			
Male	931	29 (3.1)	1.000
Female	317	10 (3.2)	
Age (yr)			
15-34	246	9 (3.7)	0.884
35-64	657	20 (3.0)	
≥ 65	344	10 (2.9)	
Unknown	1	0 (0)	
Treatment history			
New	1107	38 (3.4)	0.17
Previously treated	139	1 (0.7)	
Unknown	2	0 (0)	
Treatment outcome			
Favourable	1226	39 (3.2)	1.000
Unfavourable	23	0 (0)	
Abnormalities on x-ray			
Minor	90	8 (8.9)	0.001
Medium	590	22 (3.7)	
Major	562	9 (1.6)	
Unknown	6	0 (0)	
Smear grade[b]			
Negative or scanty	194	8 (4.1)	0.117[c]
1+	557	21 (3.8)	
2-3+	484	9 (1.9)	
Missing	13	1 (7.7)	
Duration of cough (wk)			
<4	324	10 (3.1)	
4-7	469	14 (3.0)	
≥ 8	455	15 (3.3)	
Fever			
Present	1108	36 (3.2)	0.613
Absent	140	3 (2.1)	

Table 2, continued. Patient and TB disease characteristics for patients with mixed *M. tuberculosis* infections.

Characteristic	No. (%) of patients		P value[a]
	Total	Mixed infections	
Night sweats			
Present	748	27 (3.6)	0.249
Absent	500	12 (2.4)	
Weight loss			
Present	1223	38 (3.1)	0.551
Absent	25	1 (4.0)	
Isoniazid			
Resistant	246	8 (3.3)	0.840
Sensitive	1002	31 (3.1)	
Streptomycin			
Resistant	341	15 (4.4)	0.142
Sensitive	907	24 (2.6)	
Multidrug resistant			
Yes	50	3 (6.0)	0.203
No	1198	36 (3.0)	

[a] Calculated by Fisher's exact test.
[b] The results in the reference lab; maximum grading of two smear examinations.
[c] Cuzick's test for trend, P =0.054.

Epidemiological and clinical characteristics of mixed infections

There were no significant associations between the probability of having a mixed infection and the district of residence, age, sex, treatment history, treatment delay, presence of systemic symptoms, treatment outcome, or the isolates' resistance to isoniazid or streptomycin or multidrug resistance (Table 2). However, mixed infections were significantly less likely to occur in patients with extensive X-ray abnormalities (P<0.001), and there was a nearly significant trend for lower sputum smear grades among mixed infections (P = 0.054), with both findings suggesting less extensive pathology in patients with mixed infections. Taking a medium severity of X-ray abnormalities as the reference, the odds of a mixed infection was 2.52 times (95% CI, 1.08 to 5.87) higher for minor X-ray abnormalities and 0.42 times (95% CI, 0.19 to 0.92) lower for major X-ray abnormalities. For sputum smear grading, taking a 1+ grade as the baseline, the odds of mixed infection was not higher for negative or scanty smears (1.10 times; 95% CI, 0.48 to 2.52) but was 0.48 time lower for 2+ or 3+ smears (95% CI, 0.22 to 1.07). In stratified analyses, the odds ratios for the association of mixed infection with either severity of X-ray abnormalities or sputum smear grade were not affected by any of the variables in Table 2, nor were X-ray abnormalities or sputum smear grade clearly associated with a specific genotype (EIA, Beijing, or other) among the single infections (data not shown).

On the basis of demographic information, epidemiological links between patients with mixed infections were considered highly unlikely. Patients with mixed infections were distributed equally over the three different provinces; lived in different communities; were from different sexes, age classes, and socioeconomic classes; had different types of jobs; and did not occur within the same household (data not shown).

Discussion

Our study shows that mixed tuberculosis infections also occur outside settings with extremely high TB incidences and population densities. The proportion of mixed tuberculosis infections found in rural South Vietnam in this study was 3.1% when identified by combined RFLP and spoligotyping results and 4.8% when identified by ≥ 2 loci with double alleles in VNTR typing, which are similar to the proportion reported from South Africa by Richardson *et al*. (2.3%) [14] but lower than that found in Georgia (13.1%) [15] or in South Africa by Warren *et al*. (19%) [22] and Cohen *et al*. (9%) [7]. These differences in the proportions of mixed infections could be explained by differences in the methodology of detection. The study from Georgia defined mixed infections on the basis of RFLP typing as well as VNTR typing [15]. If we would have used a definition for mixed infections adding RFLP/spoligotyping and VNTR results, our study would have identified (39 + 31)/1,248, or 5.6%, mixed infections. Moreover, a number of mixed infections in the Georgian study were detected only after typing of multiple specimens from a single patient [15], and the study by Cohen *et al*. in South Africa focused on pooled multiple samples from autopsy patients [7], while we typed only one specimen per patient. The study in South Africa by Warren *et al*. [22] used a methodology based on PCR probes distinguishing Beijing from non-Beijing strains which is more sensitive for identifying mixed infections than the RFLP typing-based method applied by Richardson *et al*. [14] that we also used. In addition, differences in the densities of human populations and risk of TB infection are likely to have played a role. The study in Georgia took place in a crowded prison with a TB incidence of 5,995/100,000, the study by Warren *et al*. was performed in an urban area with a TB incidence of as high as 1,000/100,000 [22], and the study by Cohen *et al*. was performed in a highly HIV-infected hospital population [7]. In these settings, cross-infection could easily occur. In contrast, our study was performed in a rural area in Vietnam with a population density of only 837/km2 and an observed TB incidence of new smear-positive cases of 100/100,000 in 2005 (National Tuberculosis Program Vietnam, unpublished data), and cross-infection may be expected to be less common under these circumstances. Finally, HIV infection may increase the potential for acquiring mixed TB infections [16], and the prevalence of HIV infection among TB patients was higher in the South African studies (at least 10% [22] and 94% [7]) than in ours.

To identify mixed infections in our study, we used RFLP and spoligotyping, in which strains of the EAI and Beijing genotypes can be recognized easily. It is likely that mixed infections between a Beijing and an EAI strain are easier to detect by a Beijing RFLP pattern and an EAI spoligotype than the other way around because the Beijing

RFLP pattern can mask the EAI RFLP pattern and the EAI spoligotype can mask the Beijing spoligotype. Therefore, the combination of methods is necessary to detect the mixture. The Beijing RFLP type and EAI spoligotype mixture is also the mixture that we most frequently detected in this study. We did not find any mixtures of the reverse type: an EAI RFLP type and a Beijing spoligotype. This is because an EAI strain can be visualized by RFLP typing only when the Beijing strain is present in a very low concentration compared to the EAI strain in a sample. In such a case, in spoligotyping, the EAI strain will also be amplified and visible in the spoligotyping pattern. The spoligotype of the Beijing strain that would be present in the sample would be masked by the EAI spoligotype. RFLP and VNTR typing have been used before by Shamputa *et al*. to detect mixed infections [15]. Our study showed that RFLP and spoligotyping can also be used for detection of mixed infections.

A disadvantage of our approach is that we probably underestimated the true rate of mixed infections in Vietnam. The fact that we detected 60 strains with double alleles in at least two VNTR loci suggests that VNTR typing is a more sensitive method to detect mixed infections than RFLP/spoligotyping. Neither RFLP nor spoligotyping can independently detect a mixture of two strains of the same genotype very efficiently [8], whereas VNTR typing results can show double alleles at chromosomal loci and thus in principle also detect multiple strains of the same genotype. On the other hand, 8/39 (21%) of the mixed infections were missed by VNTR typing (Table 1). We found that the more loci with double alleles an isolate showed in VNTR typing, the higher the probability was that these were also identified as mixtures by combined RFLP and spoligotyping (Fig. 2). At present, the sensitivity of VNTR typing for the detection of mixed infections is unknown, and double alleles could also represent evolution of the bacterium [13]; therefore, we did not define the mixed-infection rate according to VNTR results, but we typed all 1,248 isolates by VNTR, RFLP, and spoligotyping to compare the sensitivities of these methods to detect mixed *M. tuberculosis* infections as a first step for future studies.

With 100% of the cases being cured, we do not have an indication that mixed infections led to higher failure rates. In fact, they were associated with less extensive pulmonary pathology than single infections. Although this is based on routine chest X-ray results that were not standardized as part of our study, we found this association across all three districts, suggesting that it does not reflect observer bias. Moreover, we found a similar pattern with regard to sputum smear grading (which had been done centrally): mixed infections were associated with a lower degree of smear positivity. Interestingly, neither of these associations was determined by underlying differences in duration of cough or presence of systemic symptoms such as fever, night sweats, and weight loss. This suggests that the observed associations do not reflect early diagnosis (i.e., the patient still had limited pulmonary pathology), for example, because of a higher incidence of systemic symptoms with mixed than with single infections. This finding requires further studies; one hypothesis could be that patients with multiple infections have an increased immunological tolerance to *M. tuberculosis* infections. This may be due to HIV infection, for which we did not test. However, other causes are likely to play a role as well, since mixed infections occurred equally among both sexes

and all age groups, while HIV infection among TB patients in Vietnam is strongly associated with young age (<35 years) and male sex (18). It is also interesting that mixed-infection cases were not related to a history of TB treatment, as they occurred in 3.4% of the new patients, compared to 0.72% of the patients with recurrent TB. This finding was in agreement with the report from Georgia [15]. In contrast, Warren *et al*. [22] found that multiple infections were more frequent in relapse cases.

Although we cannot completely exclude the possibility that (part of) the mixed infections detected in our study were the result of errors or cross-contamination in the laboratory, various observations support our finding. The mixed infections were found in all three districts, with the highest frequency of mixed infections being in the district with the highest TB rate, and at different points in time. Furthermore, repeated analysis with all three DNA typing methods invariably confirmed the initially obtained results. Finally, to check for the possibility of cross-contamination, we recultured single colonies of the only two sputum specimens that were still available and could confirm mixed bacterial populations in one of these two with typing of only a very limited number of single colonies. This suggests that the observed discordant results between RFLP and spoligotyping results indeed represented mixed infections.

There were some limitations to our study. First, we did not perform HIV testing, so we could not study the relationship between HIV and mixed infections, although at 0.5% the HIV prevalence is still very low in rural Vietnam [19]. Second, we did not store all sputum samples for reculture to recheck for mixed infections. Third, the ability of RFLP and spoligotyping to detect mixtures of strains with the same genotype is limited. Fourth, VNTR typing is thought to be more sensitive than RFLP and spoligotyping to detect mixed infections, but the exact sensitivity of VNTR typing for the detection of mixed infections is unknown. Finally, the number of mixed infections that we identified was relatively small, thereby limiting the power of our study to detect significant associations with potential risk factors.

We believe that the observed 3.1% of mixed infections in the rural part of South Vietnam represents a minimum estimate, as the approach explored by combining RFLP and spoligotyping has a restricted detection limit. Most likely, due to the visual aspect involved in this approach, mixed infections that have more uneven ratios between the number of bacteria of the respective strains will go unnoticed. With the application of VNTR typing, which may be more sensitive in the detection of mixed infection, the true magnitude of this phenomenon may be unraveled in the future.

Acknowledgements

This research was funded by KNCV Tuberculosis Foundation, the Medical Committee Netherlands-Vietnam, the Netherlands, and supported by the TB adapt project (EC 6th framework project number 037919).

We thank all TB patients participating in this study and the staff of the National Tuberculosis Program of Tien Giang province for recruiting the patients as well as the staff of the National Tuberculosis Program of Pham Ngoc Thach hospital for supervision and checking data.

Dai Viet Hoa, Phan Thi Hoang Anh, and other staff in Pham Ngoc Thach laboratory are thanked for performing culture, spoligotyping, RFLP and VNTR typing. We are grateful to Anne-Marie van den Brandt, Mimount Enaimi and Arnout Mulder and other staff of the Tuberculosis Reference Laboratory at the National Institute for Public health and the Environment (The Netherlands) for providing us the knowledge and practice on the molecular techniques used in this study.

We thank the staff at the laboratory of the Oxford University Clinical Research Unit (Ho Chi Minh City) for their help in performing the VNTR typing.

References

1. Anh, D. D., M. W. Borgdorff, L. N. Van, N. T. Lan, T. van Gorkom, K. Kremer, *et al*. 2000. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 6:302-5.
2. Bates, J. H., W. W. Stead, and T. A. Rado. 1976. Phage type of tubercle bacilli isolated from patients with two or more sites of organ involvement. Am Rev Respir Dis 114:353-8.
3. Braden, C. R., G. P. Morlock, C. L. Woodley, K. R. Johnson, A. C. Colombel, M. D. Cave, *et al*. 2001. Simultaneous infection with multiple strains of *Mycobacterium tuberculosis*. Clin Infect Dis 33:e42-7.
4. Brudey, K., J. R. Driscoll, L. Rigouts, W. M. Prodinger, A. Gori, S. A. Al-Hajoj, *et al*. 2006. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 6:23.
5. Buu, T. N., M. N. Huyen, N. T. Lan, H. T. Quy, N. V. Hen, M. Zignol, *et al*. 2009. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis 13:900-6.
6. Buu, T. N., D. van Soolingen, M. N. Huyen, N. N. Lan, H. T. Quy, E. W. Tiemersma, *et al*. 2010. Tuberculosis acquired outside of households, rural Vietnam. Emerg Infect Dis 16:1466-8.
7. Cohen, T., D. Wilson, K. Wallengren, E. Y. Samuel, and M. Murray. 2010. Mixed strain *M. tuberculosis* infections among patients dying in hospital in KwaZulu-Natal, South Africa. J Clin Microbiol 49:385-8
8. de Boer, A. S., K. Kremer, M. W. Borgdorff, P. E. de Haas, H. F. Heersma, and D. van Soolingen. 2000. Genetic heterogeneity in *Mycobacterium tuberculosis* isolates reflected in IS*6110* restriction fragment length polymorphism patterns as low-intensity bands. J Clin Microbiol 38:4478-84.
9. Hoa, N. B., D. N. Sy, N. V. Nhung, E. W. Tiemersma, M. W. Borgdorff, and F. G. Cobelens. 2010. National survey of tuberculosis prevalence in Vietnam. Bull World Health Organ 88:273-80.
10. International Union Against Tuberculosis and Lung Disease. 2000. Technical guide-sputum examination for tuberculosis direct microscopy in low-income countries. 5th ed. International Union Against Tuberculosis and Lung Disease: Paris.
11. Kamerbeek, J., L. Schouls, A. Kolk, M. van Agterveld, D. van Soolingen, S. Kuijper, *et al*. 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35:907-14.

12. Kremer, K., J. R. Glynn, T. Lillebaek, S. Niemann, N. E. Kurepina, B. N. Kreiswirth, *et al*. 2004. Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. J Clin Microbiol 42:4040-9.
13. Martin, A., M. Herranz, M. J. Serrano, E. Bouza, and D. Garcia de Viedma. 2007. Rapid clonal analysis of recurrent tuberculosis by direct MIRU-VNTR typing on stored isolates. BMC Microbiol 7:73.
14. Richardson, M., N. M. Carroll, E. Engelke, G. D. Van Der Spuy, F. Salker, Z. Munch, *et al*. 2002. Multiple *Mycobacterium tuberculosis* strains in early cultures from patients in a high-incidence community setting. J Clin Microbiol 40:2750-4.
15. Shamputa, I. C., L. Jugheli, N. Sadradze, E. Willery, F. Portaels, P. Supply, *et al*. 2006. Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respir Res 7:99.
16. Small, P. M., R. W. Shafer, P. C. Hopewell, S. P. Singh, M. J. Murphy, E. Desmond, *et al*. 1993. Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. N Engl J Med 328:1137-44.
17. Supply, P., C. Allix, S. Lesjean, M. Cardoso-Oelemann, S. Rusch-Gerdes, E. Willery, *et al*. 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44:4498-510.
18. Thanh, D. H., D. N. Sy, N. D. Linh, T. M. Hoan, H. T. Dien, T. B. Thuy, *et al*. 2010. HIV infection among tuberculosis patients in Vietnam: prevalence and impact on tuberculosis notification rates. Int J Tuberc Lung Dis 14:986-93.
19. Thuy, T. T., N. S. Shah, M. H. Anh, T. Nghia do, D. Thom, T. Linh, *et al*. 2007. HIV-associated TB in An Giang Province, Vietnam, 2001-2004: epidemiology and TB treatment outcomes. PLoS One 2:e507.
20. van Embden, J. D., M. D. Cave, J. T. Crawford, J. W. Dale, K. D. Eisenach, B. Gicquel, *et al*. 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol 31:406-9.
21. van Soolingen, D., P. E. W. de Haas, and K. Kremer. 2001. Restriction fragment length polymorphism typing of mycobacteria, p. 165-203. *In* T. Parish and N. G. Stoker (ed.), In: *Mycobacterium tuberculosis* protocols. Humana Press Inc., Totowa, New Jersey, USA.
22. Warren, R. M., T. C. Victor, E. M. Streicher, M. Richardson, N. Beyers, N. C. Gey van Pittius, *et al*. 2004. Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Respir Crit Care Med 169:610-4.
23. World Health Organization. 2003. Guidelines for surveillance of drug resistance in tuberculosis. World Health Organization, Geneva, Switzerland.
24. World Health Organization. 2003. Treatment of tuberculosis. Guidelines for national programmes. World Health Organization, Geneva, Switzerland.
25. Yeh, R. W., P. C. Hopewell, and C. L. Daley. 1999. Simultaneous infection with two strains of *Mycobacterium tuberculosis* identified by restriction fragment length polymorphism analysis. Int J Tuberc Lung Dis 3:537-9.

CHAPTER 4

Tuberculosis relapse in Vietnam is significantly associated with *Mycobacterium tuberculosis* Beijing genotype infections

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The Journal of Infectious Diseases 2013; 207:1516–1524

Abstract

Background
In Vietnam, the Mycobacterium tuberculosis Beijing genotype is associated with multi-drug resistance and is emerging. A possible explanation for this genotype's success is an increased rate of relapse.

Methods
In a prospective cohort study, isolates from patients with smear-positive tuberculosis were subjected to drug susceptibility testing and to spoligotyping and variable number of tandem repeats typing before treatment and after recurrence of tuberculosis.

Results
Among 1068 patients who were actively followed up over 18 months for recurrence, 23 relapse cases occurred (1.39 cases/100 person-years). After adjustment for genotype, tuberculosis treatment history, and drug resistance, relapse was significantly associated with the Beijing genotype (adjusted hazard ratio [aHR], 5.48; 95% confidence interval [CI], 2.06–14.55) and isoniazid resistance (aHR, 5.91; 95% CI, 2.16–16.16).

Conclusions
The strongly increased relapse rate in tuberculosis cases caused by Beijing strains probably contributes to the successful spread of this genotype in Vietnam and elsewhere.

Background

Despite extensive control efforts, tuberculosis (TB) remains a major public health problem with an estimated 8.8 million cases and almost 1.4 million deaths per year worldwide [1]. On the basis of several techniques [2], molecular epidemiological studies have identified genotype families of *Mycobacterium tuberculosis*, including the Beijing genotype, which is among the predominant genotypes in East and Southeast Asia [3-6]. The Beijing genotype has gained particular attention because of its association with multi-drug resistant tuberculosis (MDR-TB), both in outbreaks and in a number of population-based studies [7-11]. In addition, its association with young adult age in several settings suggests the Beijing genotype is emerging [3,9,10]. The cause and consequence of this possible shift in the population structure of *Mycobacterium tuberculosis* has not been studied sufficiently. Although Beijing genotype strains have shown increased virulence in animal studies, data on altered pathogenesis in humans have remained inconclusive [4]. While one study from Indonesia found increased rates of treatment failure in cases caused by Beijing strains [12], other studies did not [13-16]. Reports of increased relapse rates with Beijing strains have been more consistent [14,17,18], although their study designs (case-control and/or passive follow-up) allow for alternative interpretations.

In Vietnam, the Beijing genotype is highly prevalent, causing about 40% of the TB cases [3,10], and is associated with resistance to streptomycin and isoniazid [3], as well as with MDR-TB [10], providing an ideal environment to study epidemiological phenomena associated with Beijing genotype infections. For treatment of previously untreated tuberculosis patients (new cases), the Vietnamese National Tuberculosis Program (NTP) uses an 8-month regimen with isoniazid, rifampicin, streptomycin and pyrazinamide administered for 2 months, followed by isoniazid and ethambutol for 6 months (ie, the 2HRSZ/6HE regimen). Although reported failure rates with this regimen are low (e.g. 1.7% in the south of Vietnam in 2010 [unpublished data]), there is concern about its potential role in the development of MDR-TB [5]. A study in Ho Chi Minh City found that 15 of 23 patients (65%) who did not respond to 2HRSZ/6HE treatment had acquired MDR-TB during treatment [11]. Also the rate of recurrent TB in Vietnam is high, as is the proportion of TB patients with a history of cured TB (13.3% in the south of the country, [unpublished data]). This high relapse rate may be due to use of ethambutol instead of rifampicin in the continuation phase [19], but it may also be associated with the high prevalence of the Beijing genotype among *Mycobacterium tuberculosis* isolates from previously treated patients in this country [10, 17].

We studied the association between culture-proven treatment relapse and the Beijing genotype in a prospective cohort study among patients in Vietnam with smear-positive pulmonary TB who were initially cured by first-line treatment.

Methods

Recruitment of patients and study design

This prospective cohort study was performed in three adjacent rural districts in the Mekong River Delta in southern Vietnam. Nested in a population–based study of TB cases spanning four years, the cohort included all patients consecutively diagnosed

and registered for treatment from 1 July 2005 through 30 June 2007. Details of the study site and procedures have been described elsewhere [10, 16].

In brief, all patients in the study area aged ≥15 years and with a diagnois of smear-positive pulmonary TB were eligible for enrolment and provided 2 sputum specimens for smear examination and *M. tuberculosis* culture before treatment. Enrolled patients were followed up during standard first-line treatment, with sputum smear microscopy and culture performed at the end of treatment (after 8 months). Patients whose sputum smear and culture were negative for *M. tuberculosis* were visited by study staff twice, at around 9 and 18 months after treatment completion. In addition, data were collected if patients reported during these 18 months, patients reported with symptoms at any of the participating TB clinics. Patients who had any symptom suggesting recurrent TB during these visits or who themselves consulted a participating clinic provided two sputum specimens for smear and culture. Data were also collected from clinic reports, death certificates and interviews with family members, to determine any intermediate tuberculosis treatment received elsewhere and causes of death among the study patients

Ethical clearance was obtained from the ethical health committee of the Ho Chi Minh City Council. All included patients provided written informed consent.

Laboratory procedures

Sputum specimens were kept refrigerated and transported to Pham Ngoc Thach Hospital in Ho Chi Minh City within 72 hours after collection. Specimens were decontaminated and liquefied with 1% N-Acetylcystine, 2% NaOH, inoculated on modified Ogawa medium and incubated at 37ºC. Cultures were examined for growth after 1, 2, 4, 6 and 8 weeks of incubation. Results of cultures with no growth after 8 weeks were reported as negative. *M. tuberculosis* was identified using the niacin and nitrate tests [20]. Drug susceptibility testing was performed by the proportion method [21]. Criteria for drug resistance were growth of ≥1% of the colony forming units at 28 or 42 days compared with the drug-free control medium, at the following drug concentrations: isoniazid 0.2 μg/ml, rifampicin 40 μg/ml, streptomycin 4 μg/ml and ethambutol 2 μg/ml. MDR-TB was defined as resistance to both rifampicin and isoniazid. Pretreatment drug resistance testing was performed at a later stage on stored isolates and results were not used for patient management, in accordance with Vietnamese NTP guidelines prevailing at the time of the study.

Genomic deoxyribonucleic acid (DNA) was extracted from *M. tuberculosis* positive cultures by using a previously described method [22]. Spoligotyping was performed according to the internationally standardized methods [23]. Variable Number of Tandem Repeats (VNTR) typing was done using 15 loci, as described by Supply *et al*. [24].

Definitions

We defined the cohort for the present analyses as all patients who were sputum smear- and culture-negative at the end of treatment, and we defined follow-up time as

the period between the date of completion of treatment and the date of the 18-month visit, a bacteriological diagnosis of recurrent TB, or of the patient's death, whichever came first. Recurrent TB was defined as any case in this cohort of culture-positive TB observed during the follow-up period. Unfavorable treatment outcomes were defined as recurrent TB or death during the follow-up period. Recurrent TB was considered to be due to *M. tuberculosis* re-infection if the pretreatment and follow-up isolates had different spoligotypes or VNTR types that differed by ≥ 2 loci. We defined relapse as recurrent TB caused by the same strain, with strain identity confirmed by detection of identical spoligotypes and identical VNTR profiles in pretreatment and follow-up isolates, or by detection of identical spoligotypes and VNTR types that differed by no more than a single locus [24].The Beijing genotype was defined by spoligotyping as any isolate without direct-repeat spacers 1–34 and the presence of at least 3 of the spacers 35–43 [25]. Other genotypes were defined as described by Brudey *et al*. [2].

Data analysis

Data were double entered in EpiInfo v6.04 (Centers for Disease Control and Prevention, Atlanta GA, USA); discrepancies were corrected on the basis of the raw data. Analyses were performed in Stata v10SE (Stata Corp, College Station TX, USA).

For significance testing on comparisons of categorical variables, the Chi-square test or the 2-sided Fisher's exact test was used as appropriate. Associations between genotype and outcome were assessed by survival analysis using the log-rank test. We based our primary analysis on relapse as the outcome; patients with TB re-infection were censored in this analysis.

We considered genotype, categorized as Beijing versus all other genotypes together, as our explanatory variable of interest, and considered age, sex, TB treatment and TB drug resistance as potential confounders. In similar secondary analyses, we took recurrent TB and unfavorable treatment as separate outcomes. Multivariable analyses were done by Cox proportional hazard modeling. P values for contribution to multivariate models, including interaction, were based on the likelihood ratio test. All tests were done at the 5% significance level.

Results

Of 1,331 patients enrolled in the treatment cohort, 1,073 were smear and culture negative at the end of treatment and included for follow-up in the present study. Detailed characteristics of these patients are shown in Table 1. We excluded 5 patients for loss to follow-up directly following the end of treatment (Figure 1). Of the 1,068 patients available for analysis, 984 (92.1%) initially had new TB and 84 (7.9%) had been previously treated; 816 (76.4%) were male, and 252 (23.6%) were female. Pretreatment isolates from 357 patients (33.4%) belonged to the Beijing genotype, and those from 711 patients (66.6%) belonged to other genotypes. Among these other genotypes, the most prevalent was the East African Indian genotype (411 patients, [38.5%]). Pretreatment drug resistance patterns included resistance to streptomycin in isolates from 271 patients (25.4%); resistance to isoniazid, in those from 200 (18.7%); resistance to rifampicin, in those from 24 (2.2%); resistance to ethambutol, in those

from 12 (1.1%); multi-drug resistance, in those from 22 (2.1%); and combined resistance to streptomycin and isoniazid in those from 134 (12.5%; Table 2).

The follow-up period ranged from 2.1 to 28.1 months (some patients who were not found at the 18-month visit were visited again at a later point in time), with a mean of 18.4 months. There were 20 deaths during follow-up: 1 due to TB, 2 due to accidents and 17 due to other causes. For 1 patient, no death certificate was available. Symptoms requiring collection of sputum at any time during follow-up were reported by 198 patients (18.5%). Of these, sputum samples were obtained for 185, and valid culture results were available for 181 (97.8%). Culture results were positive at any time point for 35 of 181 patients (19.3%). Of these 35 cases of recurrent TB, 12 were due to re-infection with a different strain. The 12 re-infection strains differed from the pretreatment strains by different spoligotype and VNTR type, for 3 patients and by different VNTR type, for 9.

Relapse

Relapse with the same strain was thus recorded in 23 patients (incidence rate 1.39 cases/100 person-years of follow-up). Of these, 17 patients (73.1%) had Beijing genotype infections, compared with 340 of 1,045 (32.5%) with no relapse ($p<0.001$). Survival analysis also showed a significantly higher relapse rate for patients with Beijing genotype infections than for patients with other genotypes ($p<0.001$; Figure 2). In univariate analysis, relapse was significantly associated with the Beijing genotype (hazard ratio [HR], 5.90 vs non-Beijing, $p<0.001$) as well as with resistance to streptomycin (HR, 3.19; $p=0.006$) or isoniazid (HR, 5.81; $p<0.001$). There was no significant association between relapse and history of TB treatment, age, sex, resistance to ethambutol or rifampicin or MDR-TB ($p=0.129$; Table 2). After adjustment for genotype, history of TB treatment, isoniazid resistance, streptomycin resistance and MDR, relapse remained associated with Beijing genotype (adjusted HR [HR^{adj}], 5.48; 95% confidence interval [CI], 2.06-14.55) and with isoniazid resistance (HR^{adj} 5.91; 95%CI, 2.16-16.16), but not with streptomycin resistance (Table 2).There were no significant interactions for the association with relapse between genotype and drug resistance or treatment history.

For the multivariate Cox regression model, the overall departure from the proportional hazards assumption was non-significant ($p=0.487$), whereas this departure was almost significant ($p=0.051$) for genotype. Inspection of the log-log transformed survival curves suggested convergence after 18 months of follow-up, when 1 relapse case among Beijing infections and 2 relapse cases among non-Beijing infections occurred, for equal incidence rates of 3.24 cases/100 (95%CI, <0.1-15.2) and 3.24 cases/100 person-years (95%CI, 0.3-9.6), respectively. We therefore repeated the Cox model for the first 18 months of observation only, which showed a similar pattern of associations with significant hazard ratios for Beijing genotype (HR^{adj} 7.40, 95%CI, 2.36-23.22, $p<0.001$) and isoniazid resistance (HR^{adj} 4.73, 95%CI, 1.58-14.20, $p=0.006$).

Table 1. Characteristics of 1073 patients who were cured after first-line tuberculosis treatment, Vietnam.

Characteristic	No	Percentage
Age, (yrs)		
<35	95	8.9
35-64	703	65.5
≥65	275	25.6
Sex		
Male	819	76.3
Female	254	23.7
District		
Chau Thanh	329	30.7
Cai Lay	397	37
Cai Be	347	32.3
Marital status		
Married	821	76.5
Widowed	106	9.9
Single	146	13.6
Education		
Primary school	621	57.9
Secondary school or higher	452	42.1
Profession		
Farmer or Gardener	589	54.9
Student or Salaried worker	287	26.7
House wife	49	4.6
Elderly or Jobless	148	13.8
History of tuberculosis treatment		
New cases	989	92.2
Previously treated	84	7.8
***M. tuberculosis* genotype**		
Beijing	360	33.6
East African-Indian	412	38.4
Other	301	28.0
Pretreatment drug resistance pattern		
Any streptomycin resistance	273	25.4
Any isoniazid resistance	201	18.7
Any rifampicin resistance	24	2.2
Any ethambutol resistance	12	1.1
Combined streptomycin and isoniazid resistance	135	12.6
Multidrug resistance	22	2.1

Abbreviation: *M. tuberculosis, Mycobacterium tuberculosis*.

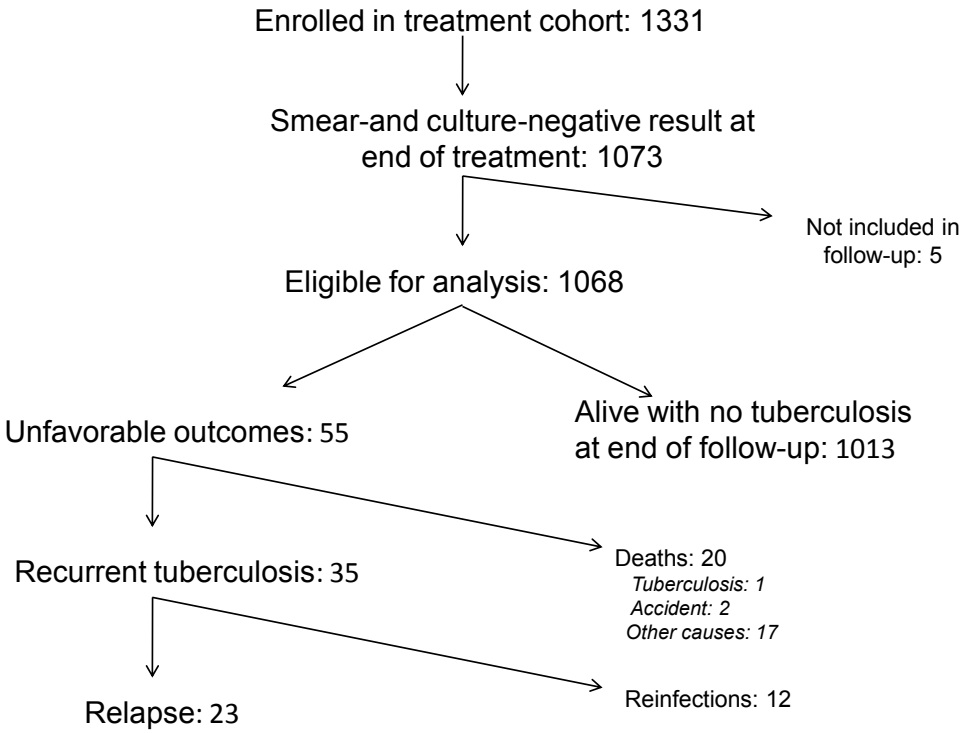

Figure 1. Study flow chart.

Recurrent TB and unfavorable outcomes

We may have misclassified relapse cases as re-infections, or as deaths. Therefore, in secondary analyses we assessed the associations between genotype and recurrent TB and unfavorable outcomes (ie, recurrent TB or death). Of 35 of 1,068 (3.3%) patients who had recurrent TB, 27 (77.1%) had Beijing genotype infections before start of treatment (difference with no recurrence, $p<0.001$). The pattern of univariate and multivariate associations for recurrent TB was similar to that for relapse (Table 3), with significant associations in the multivariate model for Beijing genotype infections (HR^{adj} 5.47; 95%CI, 2.35-12.73) and for isoniazid resistance (HR^{adj} 2.65; 95%CI, 1.10-6.34). Of the 20 patients who died during follow-up, 7 (35.0%) had Beijing genotype infections compared to 350 of 1,048 (33.4%) who survived ($p=0.880$). Unfavorable outcomes were recorded for 55 of 1,068 patients (5.1%), including 34 (61.8%) with Beijing genotype infections (difference with favorable outcomes, $p<0.001$). After adjustment for age, sex, history of TB treatment and MDR, unfavorable outcomes remained associated with Beijing genotype (HR^{adj} 3.23; 95%CI, 1.81-5.80).

Time intervals

There were no significant differences in time interval from cure to re-infection or recurrent TB between Beijing and non-Beijing genotype (19.8 versus 21.3 months, and 13.9 versus 17.2 months, respectively; $p>0.05$ for each comparison). However, the

average time from cure to relapse was shorter in patients infected with Beijing genotype strains than in patients infected with other strains (10.5 versus 15.8 months; p=0.031; Table 4).

Drug resistance amplification

Among the 21 relapse cases that had non-MDR isolates before treatment, amplification of drug resistance occurred in 4 of 15 patients (26.7%) infected by Beijing genotype strains, compared with 0 of 6 patients infected with other strains (p=0.429). Of the 4 cases involving non-MDR Beijing genotype strains in which amplification occurred, 3 had become MDR, with 1 initially monoresistant to isoniazid, and 2 initially resistant to both isoniazid and streptomycin.

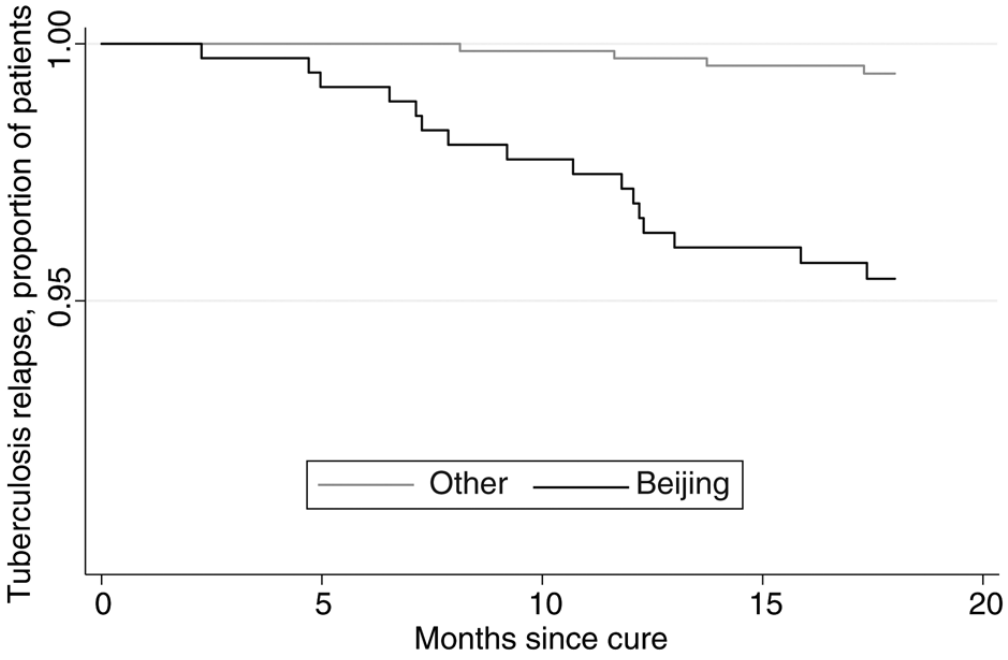

Figure 2. Survival curve for tuberculosis relapse among 1068 patients followed up after cure with first-line tuberculosis treatment in Vietnam, by genotype (other vs Beijing). P<0.001, by the log-rank test, for differences between genotypes.

Table 2. Univariate and multivariate analyses of tuberculosis relapse after cure among 1068 patients who were initially smear positive, Vietnam.

Variable	Cases, No.	Incidence, Cases/100 PYFU	Unadjusted HR	P	Adjusted HR[a]	95% CI	P
Overall	23	1.39					
Age, yrs				0.798			
<35	3	2.05	1				
35-64	14	1.32	0.64				
≥65	6	1.39	0.68				
Sex				0.446			
Male	19	1.51	1				
Female	4	1.01	0.67				
District				0.528			
Chau Thanh	5	1.00	1				
Cai Lay	10	1.68	1.76				
Cai Be	8	1.44	1.16				
History of TB treatment				0.518			0.116
New cases	22	1.44	1		1		
Previously treated	1	0.72	0.55		0.20	0.03-1.50	
***M. tuberculosis* genotype**				<0.001			<0.001
Beijing	17	3,13	5.90		5.48	2.06-14.55	
Non Beijing	6	0.54	1		1		

Table 2, continued. Univariate and multivariate analyses of tuberculosis relapse after cure among 1068 patients who were initially smear positive, Vietnam.

Variable	Cases, No.	Incidence, Cases/100 PYFU	Unadjusted HR	P	Adjusted HR[a]	95% CI	P
Pretreatment drug resistance pattern[b]							
Any streptomycin resistance	12	2.88	3.19	0.006	0.83	0.29-2.31	0.714
Any isoniazid resistance	13	4.25	5.81	<0.001	5.91	2.16-16.16	0.001
Any rifampicin resistance	2	5.26	3.53	0.150			
Any ethambutol resistance	0	0	UD				
Combined streptomycin and isoniazid resistance	9	4.38	4.47	0.001			
Multidrug resistance	2	5.76	3.84	0.129	1	0.21.-4.73	0.999

Abbreviations: CI, confidence interval; HR, hazard ratio; *M. tuberculosis*, *Mycobacterium tuberculosis*; PYFU, person-years of follow-up; UD, undetermined.

[a] Based on Cox proportional hazards modeling including all variables mentioned for the multivariate model.

[b] Reference category consists of all patients with isolates without this pattern of resistance.

Table 3. Univariate and multivariate analyses of tuberculosis recurrence after cure among 1068 patients who were initially smear positive, Vietnam.

Variable	Cases, No.	Incidence, Cases/100 PYFU	Unadjusted HR	P	Adjusted HR[a]	95% CI	P
Total	35	2.11					
Age,yrs				0.479			
<35	5	3.43	1				
35-64	22	2.04	0.52				
≥65	8	1.87	0.58				
Sex				0.463			
Male	24	1.91	1				
Female	11	2.81	1.32				
District				0.672			
Chau Thanh	9	1.81	1				
Cai Lay	14	2.35	1.43				
Cai Be	12	2.15	1.09				
History of TB treatment				0.471			0.393
New cases	31	2.04	1		1		
Previously treated	4	3.11	1.5		0.61	0.19-1.90	
***M. tuberculosis* genotype**				<0.001			<0.001
Beijing	27	4.97	6.00		5.47	2.35-12.73	
Non Beijing	8	0.72	1		1		

Table 3, continued. Univariate and multivariate analyses of tuberculosis recurrence after cure among 1068 patients who were initially smear positive, Vietnam.

Variable	Cases, No.	Incidence, Cases/100 PYFU	Unadjusted HR	P	Adjusted HR[a]	95% CI	P
Pretreatment drug resistance pattern[b]							
Any streptomycin resistance	18	4.31	2.51	0.011	0.88	0.37-2.12	0.780
Any isoniazid resistance	16	5.28	3.25	0.001	2.65	1.10-6.34	0.029
Any rifampicin resistance	4	10.51	3.89	0.037			
Any ethambutol resistance	1	5.34	2.98	0.359			
Combined streptomycin and isoniazid resistance	12	5.84	2.87	0.009			
Multidrug resistance	4	11.53	4.23	0.029	1.58	0.46-5.41	0.466

Abbreviations: CI, confidence interval; HR, hazard ratio; *M. tuberculosis, Mycobacterium tuberculosis*; PYFU, person-years of follow-up.

[a] Based on Cox proportional hazards modeling including all variables mentioned for the multivariate model.

[b] Reference category consists of all patients with isolates without this pattern of resistance.

Table 4. Interval between tuberculosis cure and recurrence, by *M. tuberculosis* genotype, among patients in Vietnam.

Event	Mean ± SD, months	Range, months	P [a]
Recurrence			
Beijing	13.9 ± 5.7	11.0-16.7	0.122
Non Beijing	17.2 ± 7.2	12.4- 21.9	
Relapse			
Beijing	10.5 ± 5.6	7.7-13.4	0.031
Non Beijing	15.8 ± 5.7	9.8-21.8	

[a] Compared with non-Beijing genotype infections.

Discussion

We observed that patients infected by Beijing genotype strains had a 5.48-times increased risk of relapse as compared to patients infected with other genotypes. This association was independent of TB treatment history and pretreatment drug resistance pattern, and was robust to possible misclassification of relapse cases as reinfections and of missed relapse cases due to death. It confirms earlier observations from Vietnam [17] and Singapore [18], as well as from a multi-country clinical trial [14], which each had limitations due to case-control design, passive follow-up and/or marginal significance of the observed difference. The relative risk of relapse for Beijing versus non-Beijing strains was larger in our study (5.48) than in the multi-country trial in which patients were also followed actively for recurrent TB (2.2) [14]. This difference may reflect the overall higher relapse rate in Vietnam that is likely associated with the use of the 8-month 2HRSZ/6HE regimen for new patients [19]. It also suggests that the effect of Beijing genotype on relapse rates may be stronger with the 8-month regimen than with the 6-month regimen, which includes a 4 month continuation phase of isoniazid-rifampicin. Furthermore, relapse in our study was significantly associated with resistance to isoniazid but not with resistance to streptomycin or MDR, probably reflecting the use of isoniazid-ethambutol in the continuation phase of treatment.

There are a number of possible explanations for the increased relapse rate in TB cases caused by Beijing genotype infections. Higher relapse rates may in fact reflect increased failure rates for Beijing genotype infections but with very low bacterial loads, resulting in negative cultures at the end of treatment. Once drug treatment is stopped these remaining bacilli start replicating again, causing symptoms and yielding positive culture results within months. This may explain why we observed shorter intervals between cure and relapse in cases caused by Beijing strains. However, our earlier study found no association between genotype and treatment failure [16], and no other study clearly did so, except for one from Indonesia [4, 12]. Alternatively, increased relapse rates may reflect increased rates of progression from latent infection to disease. In this hypothesis residual bacilli that during TB treatment become contained in granulomas, as they are during latent infection, reactivate, and Beijing bacilli do so more efficiently

than other genotype bacilli. Indeed, a study from Gambia, although based on low numbers of cases, suggested that Beijing strains have higher rates of progression from latent infection to disease [26].

The observed association of the Beijing genotype and relapse is probably due to a combination of factors. In animal models, Beijing strain bacilli escape from immune responses more efficiently than other genotype bacilli [27, 28]. Moreover, they grow more rapidly in human macrophages [29], which may provide them with a higher potential for progressing from latent infection to disease than other strains.

Note that our findings do not imply that the overall proportion of Beijing strain infections that progress to disease is increased as compared to other genotype infections. In fact, they suggest that the relapse rate for cases caused by Beijing strains is only increased during the first 18 months following cure, which would translate into a lower average incubation period rather than into a higher cumulative incidence of disease progression.

The observed increased relapse rate for Beijing strain infections was independent of (multi)drug resistance. The strong association in this site [10,16] and elsewhere [30,31] between Beijing genotype and MDR-TB implies that these increased relapse rates also result in increased spread of MDR-TB. Moreover, although differences were not significant, amplification to MDR-TB was observed for Beijing strains but not for other genotype strains in the present study. A potential explanation is provided by recent *in vitro* and mouse model data suggesting that Beijing bacteria, in comparison to the also prevalent East African Indian bacteria, have a higher intrinsic resistance to treatment by rifampicin, as well as a higher mutation frequency regarding the generation of rifampicin resistant mutants [32]. It would be interesting to subject Beijing genotype isolates that caused relapses after curative treatment to mutation analysis in fluctuation assay [32].

A higher rate of early relapse for TB cases caused by Beijing strains may explain several epidemiological observations that have thus far remained elusive. First, it would explain why, in Vietnam, the proportion of TB cases due to Beijing infections seems to increase [3,10], and why the Beijing genotype infections are more common in patients with a history of TB treatment than in new patients [10]. For Vietnam, an increased relapse rate for Beijing strains seems reason to replace the 8-month regimen by the internationally recommended 6-month regimen. Second, increased early relapse rates and/or shorter incubation periods for Beijing strains would predict that these strains have a selective advantage under conditions of very high transmission rates. This selective advantage may explain the high prevalence of Beijing strain infections in prisons and its association with history of imprisonment in former Soviet Union countries, and, in combination with a higher rate of acquisition/amplification of drug resistance, the rapid spread of MDR-TB caused by Beijing strains in these settings [8, 9].

There are potential limitations to our study. Human immunodeficiency virus (HIV) testing was not routinely performed for all patients. However, although situational data on HIV prevalence were not available, the HIV infection prevalence in Vietnam is estimated to be 0.4% of the adult population, with substantially lower

prevalence in rural provinces than in major cities [33]. Furthermore, HIV infection is not known to affect the risk of TB relapse. A recent study from Malawi showed HIV infection to be associated with an increased rate of TB re-infection but not with an increased rate of relapse, despite the use, as in Vietnam, of an 8-month first-line regimen that included rifampicin for the first 2 months only [34]. We did not collect data on the extent of radiographic abnormalities in our study patients, and some studies have suggested an association between Beijing genotype infection and pretreatment cavitation, a known risk [4]. However, the majority of published studies on this subject showed no such associations, and those that did suggest increased prevalence of cavitation among Beijing strains had no non-Beijing control group [35], found differences in radiographic patterns but not specifically for cavitation [36] or found no association after multivariate adjustment for confounding [9]. Therefore we believe that extent of pulmonary cavitation is an unlikely confounder for the association between genotype and relapse rate in our study. The follow up time for recurrence was, on average, 18 months, which may be too short to pick up all cases of TB relapse, and indeed our analyses suggested that the observed difference in relapse rates relates to this early phase only. Therefore, our findings cannot be extrapolated to longer time periods following cure. However, it is known that the recurrence of TB is highest in the first 2 years after treatment, after which it gradually decreases [37, 38].

Conclusions

Our study revealed that Beijing genotype infection is a strong and independent risk factor for relapse after first-line treatment. This may explain, at least in part, the emergence of TB due to Beijing infections in Vietnam and elsewhere.

Acknowledgements

We thank all TB patients who participated in this study and the staff of the National Tuberculosis Program of Tien Giang province for recruiting the patients as well as the staff of the National Tuberculosis Program of Pham Ngoc Thach hospital for supervision and checking data. Dai Viet Hoa, Phan Thi Hoang Anh, and other staff in Pham Ngoc Thach laboratory are deeply thanked for performing culture, spoligotyping, RFLP and VNTR typing. We are grateful to Anne-Marie van den Brandt, Mimount Enaimi and Arnout Mulder, Jessica de Beer and other staff of the Tuberculosis Reference Laboratory at the National Institute for Public Health and the Environment (The Netherlands) for providing us the knowledge and practice on the molecular techniques used in this study. We thank Dr. Maxine Caws and her staff at the laboratory of the Oxford University Clinical Research Unit (Ho Chi Minh City) for their help in performing the VNTR typing.

References

1. World Health Organization. Global tuberculosis control report 2011. WHO/HTM/TB/2011.16. Geneva: World Health Organization, 2011
2. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, *et al*. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international

spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 2006; 6:23.

3. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K, *et al*. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 2000; 6:302-5.
4. Hanekom M, Gey van Pittius NC, McEvoy C, Victor TC, Van Helden PD, Warren RM. *Mycobacterium tuberculosis* Beijing genotype: a template for success. Tuberculosis (Edinb) 2011; 91:510-23.
5. Glynn JR, Kremer K, Borgdorff MW, Rodriguez MP, Soolingen DV. Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. Emerg Infect Dis 2006; 12:736-43.
6. Filliol I, Driscoll JR, Van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G, *et al*. Global distribution of *Mycobacterium tuberculosis* spoligotypes. Emerg Infect Dis 2002; 8:1347-9.
7. Bifani PJ, Plikaytis BB, Kapur V, Stockbauer K, Pan X, Lutfey ML, *et al*. Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. JAMA 1996; 275:452-7.
8. Toungoussova OS, Sandven P, Mariandyshev AO, Nizovtseva NI, Bjune G, Caugant DA. Spread of drug-resistant *Mycobacterium tuberculosis* strains of the Beijing genotype in the Archangel Oblast, Russia. J Clin Microbiol 2002; 40:1930-7.
9. Drobniewski F, Balabanova Y, Nikolayevsky V, Ruddy M, Kuznetzov S, Zakharova S, *et al*. Drug-resistant tuberculosis, clinical virulence, and the dominance of the Beijing strain family in Russia. JAMA 2005; 293:2726-31.
10. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, *et al*. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis 2009; 13:900-6.
11. Johnson R, Warren RM, van der Spuy GD, Gey van Pittius NC, Theron D, Streicher EM, *et al*. Drug-resistant tuberculosis epidemic in the Western Cape driven by a virulent Beijing genotype strain. Int J Tuberc Lung Dis 2010; 14:119-21.
12. Parwati I, Alisjahbana B, Apriani L, Soetikno RD, Ottenhoff TH, van der Zanden AG, *et al*. *Mycobacterium tuberculosis* Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J Infect Dis 2010; 201:553-7.
13. Feng JY, Su WJ, Tsai CC, Chang SC. Clinical impact of *Mycobacterium tuberculosis* W-Beijing genotype strain infection on aged patients in Taiwan. J Clin Microbiol 2008; 46:3127-9.
14. Burman WJ, Bliven EE, Cowan L, Bozeman L, Nahid P, Diem L, *et al*. Relapse associated with active disease caused by Beijing strain of *Mycobacterium tuberculosis*. Emerg Infect Dis 2009; 15:1061-7.
15. Nahid P, Bliven EE, Kim EY, Mac Kenzie WR, Stout JE, Diem L, *et al*. Influence of *M. tuberculosis* lineage variability within a clinical trial for pulmonary tuberculosis. PLoS One **2010**; 5:e10753.
16. Buu TN, Huyen MN, van Soolingen D, Lan NT, Quy HT, Tiemersma EW, *et al*. The *Mycobacterium tuberculosis* Beijing genotype does not affect tuberculosis treatment failure in Vietnam. Clin Infect Dis 2010; 51:879-86.
17. Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerg Infect Dis 2003; 9:1633-5.

18. Sun YJ, Lee AS, Wong SY, Paton NI. Association of *Mycobacterium tuberculosis* Beijing genotype with tuberculosis relapse in Singapore. Epidemiol Infect 2006; 134:329-32.
19. Jindani A, Nunn AJ, Enarson DA. Two 8-month regimens of chemotherapy for treatment of newly diagnosed pulmonary tuberculosis: international multicentre randomised trial. Lancet 2004; 364:1244-51.
20. International Union Against Tuberculosis and Lung Disease. Technical guide-sputum examination for tuberculosis direct microscopy in low-income countries. 5th ed. Paris: The Union, 2000.
21. World Health Organization. Guideline for surveillance of drug resistance in tuberculosis. Geneva: World Health Organization, 2003.
22. van Soolingen D, de Haas PEW, Kremer K. Restriction fragment length polymorphism typing of mycobacteria. In: T. Parish, Stoker NG, eds. In: *Mycobacterium tuberculosis* protocols. Totowa, New Jersey, USA: Humana Press Inc., 2001:165-203.
23. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, *et al*. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997; 35:907-14.
24. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, *et al*. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 2006; 44:4498-510.
25. Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN, *et al*. Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. J Clin Microbiol 2004; 42:4040-9.
26. de Jong BC, Hill PC, Aiken A, Awine T, Antonio M, Adetifa IM, *et al*. Progression to active tuberculosis, but not transmission, varies by *Mycobacterium tuberculosis* lineage in The Gambia. J Infect Dis 2008; 198:1037-43.
27. Dormans J, Burger M, Aguilar D, Hernandez-Pando R, Kremer K, Roholl P, *et al*. Correlation of virulence, lung pathology, bacterial load and delayed type hypersensitivity responses after infection with different *Mycobacterium tuberculosis* genotypes in a BALB/c mouse model. Clin Exp Immunol 2004; 137:460-8.
28. Lopez B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, *et al*. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol 2003; 133:30-7.
29. Wu S, Barnes PF, Samten B, Pang X, Rodrigue S, Ghanny S, *et al*. Activation of the eis gene in a W-Beijing strain of *Mycobacterium tuberculosis* correlates with increased SigA levels and enhanced intracellular growth. Microbiology 2009; 155:1272-81.
30. Ferro BE, Nieto LM, Rozo JC, Forero L, van Soolingen D. Multidrug-resistant *Mycobacterium tuberculosis*, Southwestern Colombia. Emerg Infect Dis 2011; 17:1259-62.
31. Makinen J, MarJAMAki M, Haanpera-Heikkinen M, Marttila H, Endourova LB, Presnova SE, *et al*. Extremely high prevalence of multidrug resistant tuberculosis in Murmansk, Russia: a population-based study. Eur J Clin Microbiol Infect Dis 2011; 30:1119-26.
32. de Steenwinkel JE, Ten Kate MT, de Knegt GJ, Kremer K, Aarnoutse RE, Boeree MJ, *et al*. Drug Susceptibility of *Mycobacterium tuberculosis* Beijing Genotype and Association with MDR-TB. Emerg Infect Dis 2012; 18:660-3.

33. UNAIDS Country factsheet Vietnam. Available at: http://www.unaids.org/en/regionscountries/countries/Vietnam/. Accessed 4 October 2012
34. Crampin AC, Mwaungulu JN, Mwaungulu FD, Mwafulirwa DT, Munthali K, Floyd S, *et al*. Recurrent TB: relapse or reinfection? The effect of HIV in a general population cohort in Malawi. AIDS 2010; 24:417-26.
35. van Rie A, Warren RM, Beyers N, Gie RP, Classen CN, Richardson M, *et al*. Transmission of a multidrug-resistant *Mycobacterium tuberculosis* strain resembling "strain W" among noninstitutionalized, human immunodeficiency virus-seronegative patients. J Infect Dis 1999; 180:1608-15.
36. Feng JY, Su WJ, Liu LY, Tsai CC, Chang SC. Radiological presentation of pulmonary tuberculosis infected by the W-Beijing *Mycobacterium tuberculosis* strain. Int J Tuberc Lung Dis 2009; 13:1387-92.
37. Migliori GB, Espinal M, Danilova ID, Punga VV, Grzemska M, Raviglione MC. Frequency of recurrence among MDR-tB cases 'successfully' treated with standardised short-course chemotherapy. Int J Tuberc Lung Dis 2002; 6:858-64.
38. Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. Int J Tuberc Lung Dis 1999; 3:S231-79.

CHAPTER 5

The *Mycobacterium tuberculosis* Beijing genotype does not affect tuberculosis treatment failure in Vietnam

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Clinical Infectious Diseases 2010, 51(8):879-886

Abstract

Background
Studies have suggested that the Mycobacterium tuberculosis Beijing genotype causes more severe clinical disease and higher treatment failure rates with standard regimens, possibly in association with an increased risk of acquiring drug resistance. We studied the effect of genotype on treatment failure in a rural area in Vietnam where multidrug resistance is strongly associated with the Beijing genotype.

Methods
In a population-based prospective cohort study, patients with smear-positive tuberculosis were tested before and after treatment by spoligotyping and drug susceptibility analysis. Reinfections were excluded by DNA fingerprinting. The outcome was treatment failure based on culture.

Results
Of 1106 patients eligible for analysis, 33 experienced treatment failure (3.0%; 95% confidence interval [CI], 2.1%–4.1%). The proportion of failure was 5.3% (95% CI, 0.3%–7.9%) among 380 patients with Beijing genotype infections. Multidrug-resistant tuberculosis strongly predicted failure (odds ratio [OR], 114; 95% CI, 30–430). After adjusting for multidrug-resistant tuberculosis, treatment failure was not associated with the Beijing genotype (adjusted OR, 0.7; 95% CI, 0.3–2.0). Amplification of drug resistance occurred in 3 patients (0.3%; 95% CI, 0.1%–0.7%) and was associated with multidrug resistance at baseline (P = .004) but not with the Beijing genotype. No multidrug resistance was created.

Conclusion
The Beijing genotype was not associated with treatment failure in Vietnam; apparent associations were explained by the strong association of this genotype with multidrug resistance. Amplification of resistance in this patient population was rare.

Introduction

Despite the DOTS (directly observed treatment, short course) strategy, global tuberculosis control still faces challenges [1]. One of increasing importance is resistance of Mycobacterium tuberculosis to antituberculosis drugs. Drug resistance, especially multidrug-resistant (MDR) tuberculosis (ie, resistance to at least isoniazid and rifampin), has been a leading cause of failure with the standard first-line treatment regimens used in most high-burden countries [2–4]. Patients with MDR tuberculosis can be cured with second-line drugs but at much higher cost and with much more severe adverse effects [5–7]. In 2008, the World Health Organization (WHO) estimated that 3.6% of all incident tuberculosis cases globally are MDR, with proportions of MDR tuberculosis ranging from 0% to 28% among new and from 0% to 61.6% among previously treated patients [8].

A related threat to tuberculosis control may be the Beijing genotype. This genotype has been associated with young age [9–11], altered response to treatment [12], and drug resistance [13–16], suggesting recent emergence and increased bacterial fitness. In Vietnam, the Beijing genotype is associated with MDR tuberculosis and a history of tuberculosis treatment [17], suggesting that Beijing strain infections are more difficult to treat, leading to higher rates of treatment failure. This may be mediated by higher rates of resistance amplification, as suggested by a study from Uzbekistan [18], but it is unclear whether such an association exists irrespective of the pretreatment drug resistance pattern.

We conducted a prospective population-based cohort study to assess whether failure of standard first-line tuberculosis treatment is associated with genotype and whether this association is modified by drug resistance. A secondary objective was to assess drug resistance acquisition and amplification rates, as well as their association with genotype.

Methods

Study population and design

The study area consisted of 3 adjacent rural districts in Tien Giang Province, which is situated in the Mekong River delta in southern Vietnam at a 70-km distance from Ho Chi Minh City. The characteristics of the study site have been described elsewhere [17].

The design was a prospective cohort nested in this population- based study that collected data on all smear-positive patients given diagnoses of tuberculosis in this area between 2003 and 2007. Eligible for inclusion were all patients aged ≥15 years who were resident in the study area and registered for treatment of smear-positive pulmonary tuberculosis between 1 July 2005 and 30 June 2007 at the participating district tuberculosis units or at the provincial tuberculosis hospital. Eligible patients were included after provision of written informed consent. Excluded were patients receiving treatment for the current tuberculosis episode for >2 weeks before registration.

Diagnosis of smear-positive tuberculosis was done by microscopic examination of at least 2 Ziehl-Neelsen-stained sputum smears and followed international definitions

[19, 20]. All included patients were requested to submit 2 sputumspecimens that were sent for culture.

We performed sputum cultures for all enrolled patients who completed treatment (ie, after 8 months). Since in DOTS treatment these patients are considered to have experienced failure and stop treatment, we also performed cultures for new patients who had a positive sputum smear result at 5 months. Patients submitted 2 sputum samples on 2 consecutive days, including at least 1 morning sample, after receiving instruction on effective expectoration.

Tuberculosis was treated in accordance with the guidelines of the Vietnam National Tuberculosis Control Program [21]. Patients not previously treated for tuberculosis (new patients) were treated with 2 months of daily streptomycin (S), isoniazid (H), rifampin (R), and pyrazinamid (Z; intensive phase), followed by daily ethambutol (E) and isoniazid for 6 months (continuation phase; category 1 regimen). Previously treated patients were given all 5 drugs (SHRZE) daily for 2 months, then 4 drugs (HRZE) for 1 more month followed by RHE 3 days per week for 5 months (category 2 regimen). All doses were given under direct observation as long as patients were given rifampin, irrespective of treatment phase or regimen.

Scientific and ethical clearance was obtained from the Ethical Health Committee of the Ho Chi Minh City Council. Pretreatment drug resistance testing was done later and was not used to guide treatment decisions. All patients, including those identified with MDR infections, received treatment following the guidelines of the National Tuberculosis Control Program of Vietnam.

Based on an expected 6.5% culture-based failure rate and a prevalence of Beijing genotype infections in this population of 35% [4, 17], we calculated that enrollment of 1023 patients would be sufficient to detect a 2-fold increased failure rate among Beijing genotype infections at 80% power.

Laboratory methods

Sputum samples were kept refrigerated and transported to the Tuberculosis Reference Laboratory in Ho Chi Minh City within 7 days. At the laboratory, 1 smear examination (Ziehl-Neelsen staining) was performed for each specimen. Sputum specimens were subsequently decontaminated and liquefied with 1% N-acetyl-L-cysteine-2% NaOH, inoculated on modified Ogawa medium, and incubated at 37°C [22]. Cultures with no growth after 8 weeks were reported as negative. M. tuberculosis was identified by the niacin and nitrate tests. Drug susceptibility testing was done by the proportion method, in accordance with WHO/International Union Against Tuberculosis and Lung Disease guidelines. Criteria for drug resistance were $\geq$1% colony growth at 28 or 40 days compared with that in the drug-free control medium at the following drug concentrations: isoniazid, 0.2 µg/mL; rifampin, 40 µg/mL; streptomycin, 4 µg/mL; and ethambutol, 2 µg/mL [22]. Standard operational procedures and checks on consecutively identified strains by resistance pattern and genotype were used to prevent and detect cross-contamination.M. tuberculosis isolates were genotyped by spoligotyping using the standardized method [23] and were fingerprinted by IS6110-based restriction fragment-length polymorphism (RFLP) typing and 15-loci variable

number of tandem repeat (VNTR) typing, to exclude reinfection and laboratory cross-contamination [24–27].

Definitions

Previously treated patients were defined as having been treated with antituberculosis drugs for ≥1 month; otherwise, participants were deemed to be new patients. Treatment failure occurred if a patient had (1) a positive sputum smear and/or culture result after 5 months or at the end of the 8th month of category 1 treatment or (2) a positive sputum smear and/or culture result at the end of the 8th month of category 2 treatment [19].

The Beijing genotype was defined by spoligotyping as any isolate without direct-repeat spacers 1–34 and the presence of ≥3 of the spacers 35–43 [25]. Other genotypes were defined as described by Brudey *et al*, including the Vietnam genotype (EAI-VNM) that belongs to the East African Indian genotype family of M. tuberculosis and that is the most frequent genotype in this study site [17, 26]. Treatment adherence was ascertained from the routine treatment cards kept at the district tuberculosis unit. Adherence to treatment was defined as regular if a patient had taken antituberculosis drugs regularly both in the intensive and continuation phase. Adherence was defined as irregular for those patients who had not taken antituberculosis drugs regularly. Positive culture results for follow-up samples were considered due to reinfection if the pretreatment and follow-up isolate had different spoligotypes or if they had identical spoligotypes but either different RFLP patterns or VNTR types that differed for ≥2 loci [27].

Data management

Data were double-entered in EpiInfo software, version 6.04 (Centers for Disease Control and Prevention); discrepancies were corrected on the basis of the raw data. Analyses were performed in Stata software, version 8 (StataCorp). Patients with negative culture results or cultures that grew nontuberculosis mycobacteria, as well as cases of reinfection and changes of drug regimen during treatment, were excluded from the analyses of failure and resistance acquisition and amplification. In these analyses, treatment failure was based on the culture results only.

For significance testing of comparisons of categorical variables, either the χ^2 test or the 2-sided Fisher exact test was used as appropriate. Multivariate analyses were done by logistic regression modeling. P values for contributing to multivariate models, including interaction, were based on the likelihood ratio χ^2 test; P values for contributing to models of individual strata of variables were based on the Wald test. All tests were done at the 5% significance level.

Results

During the study period, 1364 patients with smear-positive pulmonary tuberculosis were registered for treatment, and 1331 enrolled in the study (Figure 1). Pretreatment culture results were available for 1295 patients; 1213 of the cultures grew M. tuberculosis and were subjected to drug susceptibility testing and molecular typing.

Of the 1213 patients who were finally included in the cohort analysis, we excluded 95, because of death (49), loss of data (32), defaulting (10) and transferring out (4); therefore, culture results were available for 1118 (92.2%). Of the 1118 remaining patients, 41 had positive follow- up culture results (3.7%). Of these, 8 patients were classified as having reinfections (including 5 infected with Beijing genotype strains) and 4 had changed treatment regimen; these patients were also excluded from the final analysis. No case of cross-contamination was found.

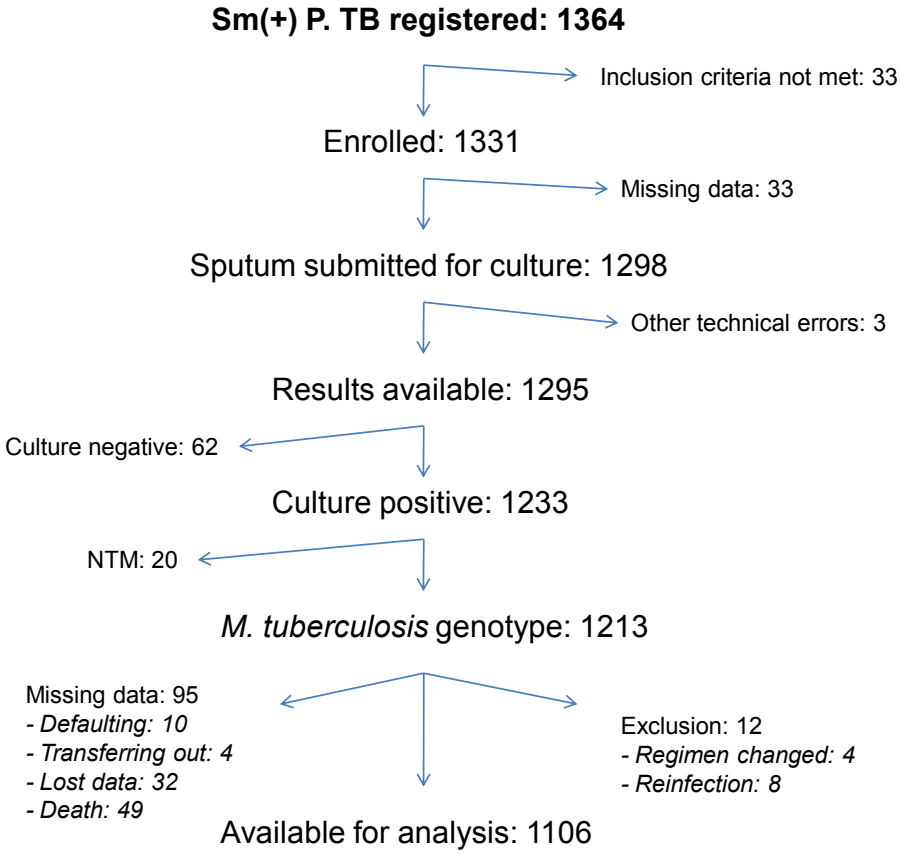

Figure 1. Study enrollment and inclusion and exclusion of patients. NTM, nontuberculosis mycobacteria; Sm (+) P.TB, smear-positive pulmonary tuberculosis.

Of the 1106 patients eligible for analysis, 1010 were new (91.3%) and 96 were previously treated. The prevalence of Beijing genotype infections was 34.4% (380/1106) overall, 31.8% (321/1010) among new patients, and 61.5% (59/96) among previously treated patients ($P < .001$). Treatment failure was recorded in 23 patients (2.1%; 95% confidence interval [CI], 1.4%–3.0%) on the basis of direct smear microscopy, in 33 patients (3.0%; 95% CI, 2.1%–4.1%) on the basis of culture, and in 39 patients (3.5%; 95% CI, 2.6%–4.7%) on the basis of either. MDR tuberculosis was strongly associated with the Beijing genotype: 17 (5.3%) of 321 versus 5 (0.7%) of 689

among new cases (risk ratio, 7.5; P < .001) and 17 (28.8%) of 59 versus 3 (8.1%) of 37 among previously treated cases (risk ratio, 3.6; P = .019).

In univariate analysis, treatment failure was significantly associated with infection with the Beijing genotype (odds ratio [OR] vs non-Beijing, 2.9). Failure was also associated with MDR (OR vs pansusceptible to all first-line antituberculosis drugs tested, 106), with drug resistance other than MDR (OR vs pansusceptible to all first-line antituberculosis drugs tested, 5.1), and with history of tuberculosis treatment (OR vs new patients, 6.0) (Table 1).

After adjustment for drug resistance, treatment history, treatment adherence, and demographic factors (Table 1), treatment failure remained associated with MDR (adjusted OR, 114; 95% CI, 30–431) but did not remain associated with the Beijing genotype (adjusted OR, 0.71; 95% CI, 0.3–2.0) or with a history of tuberculosis treatment (adjusted OR, 1.7; 95% CI, 0.6–5.4) (Table 1). We found no interaction between genotype and other variables that affected failure.

To investigate the apparent confounding effect of MDR on the association between failure and genotype, we compared the various combinations of genotype and MDR, taking non-MDR/ non-Beijing genotype infections as the reference stratum (Table 2). Among new patients, the odds of failure for non-MDR/ Beijing genotype infections was not increased (OR, 0.8; 95% CI, 0.2–3.0), whereas it was strongly and similarly increased for MDR/non-Beijing (OR, 50.0; 95% CI, 5.1–447) and for MDR/ Beijing genotype infections (OR, 52.5; 95% CI, 14.2–198). Among previously treated patients, the corresponding ORs were 0 (95% CI, 0.0–14.3), 16.5 (95% CI, 0.0–1122), and 47.1 (95% CI, 4.7–1161).

Since death during treatment may be a consequence of treatment failure, we conducted a secondary analysis taking failure and death as the combined outcome. The association with the Beijing genotype remained unchanged (adjusted OR, 0.8; 95% CI, 0.6 –1.5). Because the proportion of Beijing genotype infection among reinfected patients was high, we also reincluded all reinfected cases. This slightly increased the relative risk of failure for Beijing infections, but it remained small and non-significant (adjusted OR, 1.26; 95% CI, 0.6 – 2.9).

Amplification of tuberculosis drug resistance was recorded in 3 patients (0.3%; 95% CI, 0.1% – 0.7%), including one of 1064 with initially non-MDR strains (0.1%) and 2 of 42 with initially MDR strains (4.8%, P=0.004). The amplification rates among new and previous treated patients were 0.2% and 1.0%, respectively (Table 3), and were similar between patients infected with the Beijing genotype and patients infected with other strains (risk ratio 0.95; P>0.99).

Among new patients, 1 case of acquired drug resistance occurred from an initially pansusceptible strain to monoresistance to isoniazid. One additional non-Beijing strain developed resistance to all 4 drugs (SHRE) from initial MDR (RHE). Of 12 cases of failure during category 2 treatment, 1 strain (Beijing genotype) amplified resistance to all 4 drugs (RHES) from initial resistance to RHS.

Table 1. Univariate and multivariate associations with treatment failure versus cure among patients with smear-positive pulmonary tuberculosis in rural Vietnam.

Category, parameter	All	Failure n (%)[a]	Crude OR[b]	Adjusted OR [c] (95% CI)	P [d]
All	1106	33 (3.0%)			
District					0.028
Chau Thanh	338	9 (2.7%)	1	1	
Cai Lay	403	5 (1.2%)	0.5	0.1 (0.1 – 1.5)	
Cai Be	365	19 (5.2%)	2.0	2.0 (0.8 – 5.1)	
Age					0.724
<35 years	255	15 (5.9%)	2.7	1.7 (0.4 – 7.1)	
35 – 64 years	589	12 (2.0%)	0.9	1.5 (0.5 – 5.0)	
≥65 years	262	6 (2.3%)	1	1	
Sex					0.814
Male	843	23 (2.7%)	1	1	
Female	263	10 (3.8%)	1.4	0.89 (0.3 – 2.4)	
Marital status					0.500
Married	843	22 (2.6%)	1	1	
Widowed/Divorced	108	2 (1.9%)	0.7	0.95 (0.2-5.0)	
Single	155	9 (5.8%)	2.3	2.1 (0.6 – 7.0)	
Genotype					0.509
Non-Beijing	726	13 (1.8%)	1	1	
Beijing	380	20 (5.3%)	3.1	0.7 (0.3 – 2.0)	
History of tuberculosis					0.348
New patients	1010	21 (2.1%)	1	1	
Previously treated patients	96	12 (12.5%)	6.0	1.7 (0.6 – 5.4)	
Drug susceptibity testing					<0.001
Pansusceptible [e]	944	8 (0.9%)	1	1	
Drug resistant, not MDR	120	5 (4.2%)	5.1	2.6 (0.8 – 8.1)	
Multidrug resistant	42	20 (47.6%)	106.4	114 (30 – 431)	
Treatment adherence					0.801
Regular	931	29 (3.1%)	1	1	
Irregular	175	4 (2.3%)	0.7	1.2 (0.4 – 4.0)	

Note. CI, confidence interval; OR, odds ratio; MDR: multidrug resistant

[a] Row percentages.

[b] Unadjusted ORs.

[c] ORs adjusted by logistic regression modelling for all other variables in the model.

[d] *P* value for multivariable association.

[e] With all first line drugs tested

Table 2. Association of multidrug-resistant (MDR) tuberculosis status and genotype with treatment failure among patients with smear-positive pulmonary tuberculosis.

MDR tuberculosis status and genotype	All	Failure n (%)	OR(95% CI)	P
All smear positive	1106	33 (3.0)		
Non MDR/non-Beijing	718	10 (1.4)	1	
Non MDR/Beijing	346	3 (0.9)	0.6 (0.1 – 2.5)	>0.05[a]
MDR/non-Beijing	8	3 (37.5)	42.5 (6.9 – 250.7)	<0.05[a]
MDR/Beijing	34	17 (50.0)	70.8 (26.0 – 197.6)	<0.001
New patients	1010	21 (2.1)		
Non MDR/non-Beijing	684	9 (1.3)	1	
Non MDR/Beijing	304	3 (10)	0.8 (0.2 – 3.0)	>0.05[a]
MDR/non-Beijing	5	2 (40.0)	50.0 (5.1 – 446.7)	<0.05[a]
MDR/Beijing	17	7 (41.2)	52.5 (14.2 – 197.8)	<0.001
Previously treated patients	96	12 (12.5)		
Non MDR/non-Beijing	34	1 (2.9)	1	
Non MDR/Beijing	42	0 (0.0)	0.0 (0.0 – 14.3)	>0.05[a]
MDR/non-Beijing	3	1 (33.3)	16.5 (0.0 – 1122.8)	>0.05[a]
MDR/Beijing	17	10 (58.8)	47.1 (4.7 – 1161.1)	<0.001[a]

Note. MDR, multidrug resistant; CI, confidence interval; OR, odds ratio.
[a] Fisher exact test.

Discussion

In this study, treatment failure was strongly associated with MDR, whereas genotype, especially the Beijing genotype, was not an independent predictor of failure. Because the prevalence of drug resistance and MDR was considerably higher among the Beijing genotype than among other genotypes, drug resistance and MDR confounded the association between genotype and treatment failure: the univariate association between Beijing genotype and treatment failure completely disappeared after adjustment for differences in pretreatment drug resistance and other patient characteristics. Moreover, we found no interactions—that is, the effect of genotype on treatment failure did not vary by drug resistance pattern or treatment history. Therefore, the probability of cure or treatment failure is equal among patients infected with strains with the same pretreatment pattern of tuberculosis drug resistance irrespective of whether the strain is of the Beijing genotype.

Table 3. Drug resistance pattern before treatment and at failure, confirmed by sputum culture

Pretreatment drug resistance pattern	Total no.	No. with failure	Drug resistance pattern in patients with treatment failure						
			Pan-susceptible	Mono-resistance to H	Mono-resistance to S	Resistance to HS	Resistance to HES	Resistance to RHS	Resistance to HSRE
New patients	1010	21							
Pansusceptible[a]	705	6	5	1[b]	0	0	0	0	0
Monoresistance to H	51	0	0	0	0	0	0	0	0
Monoresistance to S	127	1	0	0	1	0	0	0	0
Monoresistance to R	2	0	0	0	0	0	0	0	0
Resistance to HS	100	4	0	0	0	4	0	0	0
Resistance to HES	3	1	0	0	0	0	1	0	0
Resistance to RHE	2	1	0	0	0	0	0	0	1[b]
Resistance to RHS	12	4	0	0	0	0	0	4	0
Resistance to HSRE	8	4	0	0	0	0	0	0	4
Previously treated patients	96	12							
Pansusceptible[a]	33	0	0	0	0	0	0	0	0
Monoresistance to H	14	1	0	1	0	0	0	0	0
Monoresistance to S	12	0	0	0	0	0	0	0	0
Resistance to HS	15	0	0	0	0	0	0	0	0
Resistance to HES	2	0	0	0	0	0	0	0	0
Resistance to RH	1	0	0	0	0	0	0	0	0
Resistance to RHS	10	5	0	0	0	0	0	4	1[b]
Resistance to HSRE	9	6	0	0	0	0	0	0	6

Note. E, ethambutol; H, isoniazid; R, rifampin; S, streptomycin.

[a] With all first-line drugs tested.

[b] Resistance amplification.

Our findings are similar to those of a smaller study conducted in Taiwan [28] but are different from those of a study conducted in Ho Chi Minh City, Vietnam [29], possibly because that study had too few patients to distinguish the effect of genotype on failure from that on relapse; there might still be a difference in relapse rates between different genotypes [30]. Indeed, if Beijing genotype strains were more likely to manifest a slower response to treatment, the relapse rate could be more affected than the failure rate.

Our findings also differ from those of a recent hospital-based cohort study conducted in Indonesia that, after adjustment for drug resistance, observed a 1.9 times higher failure rate (P = .04) for infections with the Beijing genotype comparedwith other genotypes [31]. That study had resistance data for only 59% of enrolled patients and did not exclude reinfections, which may have lead to incomplete adjustment for confounding.

Alternatively, the differences between these study results may reflect the low failure rates in our study (3.0%) compared with those in Ho Chi Minh City (4.3% on the basis of smear examination only) [29] and Indonesia (8.8%) [31]. This suggests a high level of treatment adherence in this rural site, possibly limiting the risk of failure as well as of resistance amplification.

Indeed, acquisition or amplification of tuberculosis drug resistance during treatment (0.3% overall and none to MDR) was less than expected. In Uzbekistan, amplification occurred in 1.2% of patients with initially susceptible or monoresistant strains but in 17% of patients with initially polyresistant strains [18]. That study also suggested a higher risk of amplification among Beijing strains than among non-Beijing strains, whereas we did not observe such difference. The differences between that study and ours could relate to differences in treatment regimens used for new patients. Whereas in our study the continuation phase consisted of daily isoniazid and ethambutol for 6 months, in Uzbekistan this was 4 months of rifampin and isoniazid (with ethambutol added in the case of baseline rifampin or isoniazid resistance), and dosing was thrice weekly. This long period of rifampin in combination with the intermittent dosing could enhance amplification of resistance to rifampin, in particular if combined resistance to isoniazid and ethambutol or streptomycin is already present [32]. The study from Ho Chi Minh City also reported higher amplification rates: 65% of patients who experienced treatment failure without MDR tuberculosis at baseline underwent amplification to MDR tuberculosis [33]. Although the same treatment regimen was used as in our study, poorer treatment adherence in Ho Chi Minh City relative to that in the rural Mekong River delta could be a possible explanation [9, 34]. In addition, our study was underpowered to detect an effect of genotype on amplification of drug resistance.

Taken together, these observations suggest that the Beijing genotype could be a risk factor for resistance amplification and treatment failure, but only if adherence to tuberculosis treatment is poor or treatment is given intermittently.

In one-fifth of the patients with treatment failure, the posttreatment strain differed from the strain isolated before treatment, suggesting that reinfection is common in Vietnam as it is in other areas in which the incidence of tuberculosis is high [35, 36].

This high rate of reinfection may, however, also reflect the presence of multiple infections by different strains before start of treatment, which has also been observed in other highincidence settings [37]. An infecting strain that remained undetected by our typing methods because of low numbers of bacilli in the pretreatment sputum specimen may have been detected as the posttreatment strain, in particular when it was drug resistant (Table 3) [38]. In such a case there would be treatment failure rather than reinfection, and we cannot exclude that this occurred. However, given that the study question was whether genotype affects treatment outcome irrespective of pretreatment drug resistance and that half of the patients with treatment failure were infected with pansusceptible strains, this is unlikely to have affected our conclusions.

Our study had some other limitations. First, the failure rate in our study was lower than assumed, thus limiting the statistical power for finding an increased failure rate with Beijing genotype infections if it existed. Nonetheless, the adjusted relative risk of failure with Beijing genotype infections was in fact <1, and the probability of observing our data if the true relative risk was 1.9 (as in the Indonesian study) was <2.5%. Second, we did not follow-up for relapse after treatment. Third, our data are representative only of patients with smear-positive pulmonary tuberculosis, which limits the generalizability of our findings to smear-negative and extrapulmonary tuberculosis. Next, we did not test for human immunodeficiency virus (HIV) infection, while a positive association between HIV infection and the Beijing genotype has been suggested for tuberculous meningitis [38]. However, HIV infection is not known to affect treatment failure rates for pulmonary tuberculosis [39], making it unlikely that HIV infection would have confounded the association between genotype and failure. Finally, sputum collection, transport conditions, and potential overdecontamination (given that culture contamination was not observed) could have affected the sensitivity of culture at the end of treatment, resulting in underestimation the failure rate. Because this is unlikely to have been different between genotypes, it would have lead to reduced statistical power but not to bias of the relative risk estimate [40] – that is, it would not have affected our conclusions.

In conclusion, failure of tuberculosis treatment with a standard first-line regimen was strongly associated with multidrug resistance but not with the Beijing genotype. Neither was the Beijing genotype associated with amplification of tuberculosis drug resistance. Our study suggests that the Beijing genotype does not affect tuberculosis treatment outcomes in settings with good treatment adherence and low prevalence of drug resistance and MDR.

Acknowledgments

We thank the tuberculosis staff working at the district tuberculosis units of Cai Be, Cai Lay, and Chau Thanh for collecting data and tracing patients at home under difficult working conditions and the staff at the National Institute of Public Health and the Environment, the Netherlands, for technical support in processing and transcription of the VNTR results.

Financial support was provided by KNCV Tuberculosis Foundation, the Medical Committee Netherlands-Vietnam, and the World Health Organization.

References

1. Dye C. Global epidemiology of tuberculosis. Lancet 2006; 367:938-9.
2. Amnuaiphon W, Anuwatnonthakate A, Nuyongphak P, Sinthuwatanawibool C, Rujiwongsakorn S, Nakara P, *et al*. Factors associated with death among HIV-uninfected TB patients in Thailand, 2004-2006. Trop Med Int Health 2009; 14:1338-46.
3. Singla R, Srinath D, Gupta S, Visalakshi P, Khalid UK, Singla N, *et al*. Risk factors for new pulmonary tuberculosis patients failing treatment under the Revised National Tuberculosis Control Programme, India. Int J Tuberc Lung Dis 2009; 13:521-6.
4. Quy HT, Cobelens FG, Lan NT, Buu TN, Lambregts CS, Borgdorff MW. Treatment outcomes by drug resistance and HIV status among tuberculosis patients in Ho Chi Minh City, Vietnam. Int J Tuberc Lung Dis 2006; 10:45-51.
5. Kwon YS, Kim YH, Suh GY, Chung MP, Kim H, Kwon OJ, *et al*. Treatment outcomes for HIV-uninfected patients with multidrug-resistant and extensively drug-resistant tuberculosis. Clin Infect Dis 2008; 47: 496-502.
6. Masjedi MR, Tabarsi P, Chitsaz E, Baghaei P, Mirsaeidi M, Amiri MV, *et al*. Outcome of treatment of MDR-TB patients with standardised regimens, Iran, 2002-2006. Int J Tuberc Lung Dis 2008; 12:750-5.
7. Ferrara G, Richeldi L, Bugiani M, Cirillo D, Besozzi G, Nutini S, *et al*. Management of multidrug-resistant tuberculosis in Italy. Int J Tuberc Lung Dis 2005**;** 9:507-13.
8. World Health Organization. Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. Geneva: World Health Organization, 2010.
9. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K, *et al*. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 2000; 6:302-5.
10. Borgdorff MW, de Haas P, Kremer K, van Soolingen D. *Mycobacterium tuberculosis* Beijing genotype, the Netherlands. Emerg Infect Dis 2003; 9:1310-3.
11. Jou R, Chiang CY, Huang WL. Distribution of the Beijing family genotypes of *M. tuberculosis* in Taiwan. J Clin Microbiol 2005; 43:95-100.
12. van Crevel R, Nelwan RH, de Lenne W, Veeraragu Y, van der Zanden AG, Amin Z, *et al*. *Mycobacterium tuberculosis* Beijing genotype strains associated with febrile response to treatment. Emerg Infect Dis 2001; 7:880-3.
13. Cox HS, Kubica T, Doshetov D, Kebede Y, Rüsch-Gerdess S, Niemann S. The Beijing genotype and drug resistant tuberculosis in the Aral Sea region of Central Asia. Respir Res 2005; 6:134.
14. Almeida D, Rodrigues C, Ashavaid TF, Lalvani A, Udwadia ZF, Mehta A. High incidence of the Beijing genotype among multidrug-resistant isolates of *M. tuberculosis* in a tertiary care center in Mumbai, India. Clin Infect Dis 2005; 40:881-6.
15. Balabanova Y, Drobniewski F, Fedorin I, Zakharova S, Nikolayevskyy V, Atun R,*et al*. The Direct Observed Therapy Short course (DOTS) strategy in Samara Oblast, Russian Federation. Respir Res 2006; 7:44.
16. Sun YJ, Lee AS, Wong SY, Heersma H, Kremer K, van Soolingen D,*et al*. Genotype and phenotype relationships and transmission analysis of drug-resistant tuberculosis in Singapore. Int J Tuberc Lung Dis 2007; 11:436-42.
17. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, *et al*. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis 2009; 13:900-6.
18. Cox HS, Niemann S, Ismailov G, Doshetov D, Orozco JD, Blok L, *et al*. Risk of acquired

drug resistance during short course directly observed treatment of tuberculosis in an area with high level of drug resistance. Clin Infect Dis 2007; 44:1421-37.

19. World Health Organization. Treatment of Tuberculosis. Guidelines for national programmes. Geneva: World Health Organization, 2003.
20. International Union Against Tuberculosis and Lung Disease. Technical Guide – Sputum examination for tuberculosis direct microscopy in low income countries. 5th ed. Paris: The Union, 2000.
21. Huong NT, Duong BD, Co NV, Quy HT, Tung LB, Bosman M, *et al*. Establishment and development of the National Tuberculosis Control Programme in Vietnam. Int J Tuberc Lung Dis 2005; 9:151-6.
22. World Health Organization. Guidelines for surveillance of drug resistance in tuberculosis. Geneva: World Health Organization, 2003.
23. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, *et al*. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997; 35:907-14.
24. Institute de Biologie – Institute Pateur the Lille. Multilocus variable number tandem repeat genotyping of *Mycobacterium tuberculosis*. Technical guide. Lille: Institut Pasteur, 2005.
25. Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN, *et al*. Definition of the Beijing/W lineage of *M. tuberculosis* on the basis of genetic markers. J Clin Microbiol 2004; 42:4040-9.
26. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, *et al*. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 2006; 6:23.
27. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rüsch-Gerdes S, Willery E, *et al*. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 2006; 44:4498-510.
28. Feng JY, Su WJ, Tsai CC, Chang SC. Clinical Impact of *Mycobacterium tuberculosis* W-Beijing Genotype Strain Infection on Aged Patients in Taiwan. J Clin Microbiol 2008; 46:3127-9.
29. Lan NT, Lien HT, Tung LB, Borgdorff MW, Kremer K, van Soolingen D. *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerg Infect Dis 2003; 9:1633-5.
30. Parwati I, van Crevel R, Sudiro M, Alisjahbana B, Pakasi T, Kremer K, *et al*. *Mycobacterium tuberculosis* population structures differ significantly on two Indonesian Islands. J Clin Microbiol 2008; 46:3639-45.
31. Parwati I, Alisjahbana B, Apriani L, Soetikno RD, Ottenhoff TH, van der Zanden AG, *et al*. *Mycobacterium tuberculosis* Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J Infect Dis 2010; 201:553-7.
32. Lew W, Pai M, Oxlade O, Martin D, Menzies D. Initial drug resistance and tuberculosis treatment outcomes: systematic review and meta-analysis. Ann Intern Med 2008;149:123-34.
33. Quy HT, Lan NT, Borgdorff MW, Grosset J, Linh PD, Tung LB, *et al*. Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate? Int J Tuberc Lung Dis 2003; 7:631-6.

34. Lönnroth K, Thuong LM, Lambregts K, Quy HT, Diwan VK. Private tuberculosis care provision associated with poor treatment outcome: comparative study of a semi-private lung clinic and the NTP in two urban districts in Ho Chi Minh City, Vietnam. National Tuberculosis Programme.Int J Tuberc Lung Dis 2003; 7:165-71.
35. Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, Gey van Pittius NC, *et al*. Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Respir Crit Care Med 2004; 169:610-4.
36. Wang JY, Lee LN, Lai HC, Hsu HL, Liaw YS, Hsueh PR, *et al*. Prediction of the tuberculosis reinfection proportion from the local incidence. J Infect Dis 2007; 196:281-8.
37. Verver S, Warren RM, Beyers N, Richardson M, van der Spuy GD, Borgdorff MW, *et al*. Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis.Am J Respir Crit Care Med 2005; 171:1430-5.
38. Caws M, Thwaites G, Stepniewska K, Nguyen TN, Nguyen TH, Nguyen TP, *et al*. Beijing genotype of *Mycobacterium tuberculosis* is significantly associated with human immunodeficiency virus infection and multidrug resistance in cases of tuberculous meningitis. J Clin Microbiol 2006; 44:3934-9.
39. Havlir DV, Barnes PF. Tuberculosis in patients with human immunodeficiency virus infection. N Engl J Med 1999; 340:367-73.
40. White E. The effect of misclassification of disease status in follow-up studies: implications for selecting disease classifiaction criteria. Am J Epidemiol 1986; 124:816-25.

CHAPTER 6

Epidemiology of isoniazid resistance mutations and their effect on tuberculosis treatment outcomes

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Antimicrobial Agents and Chemotherapy 2013, 57(8):3620-3627.

Abstract

Background
Isoniazid resistance is highly prevalent in Vietnam. We investigated the molecular and epidemiological characteristics and the association with first-line treatment outcomes of the main isoniazid resistance mutations in *Mycobacterium tuberculosis* in codon 315 of the *katG* and in the promoter region of the *inhA* gene.

Methods
Mycobacterium tuberculosis strains with phenotypic resistance to isoniazid from consecutively diagnosed smear-positive tuberculosis patients in rural Vietnam were subjected to Genotype® MTBDR*plus* testing to identify *katG* and *inhA* mutations. Treatment failure and relapse were determined by sputum culture.

Results
In total 227of 251 isoniazid-resistant strains (90.4%) had detectable mutations: 75.3% in the *katG*315 codon and 28.2% in the *inhA* promoter region. *KatG*315 mutations were significantly associated with pre-treatment resistance to streptomycin, rifampicin and ethambutol, but not with the Beijing genotype, and predicted both unfavorable treatment outcome (treatment failure or death) and relapse *inhA* promoter region mutations were only associated with resistance to streptomycin and relapse.

Conclusions
In tuberculosis patients, *M. tuberculosis katG*315 mutations but not *inhA* mutations are associated with unfavorable treatment outcome. *InhA* mutations do, however, increase the risk of relapse, at least with treatment regimens that contain only isoniazid and ethambutol in the continuation phase.

Introduction

With 8.8 million cases notified and 1.4 million deaths in 2010, tuberculosis (TB) remains a major burden to global health [1]. In addition to rifampicin, isoniazid is an important drug in first-line anti-tuberculosis treatment [2]. *Mycobacterium tuberculosis* (MTB) strains resistant to at least both rifampicin and isoniazid are referred to as multi-drug resistant (MDR). Both multidrug resistance and resistance to isoniazid without concomitant rifampicin resistance are associated with poor response to first-line treatment [3,4]. Whereas rifampicin resistance is usually encoded in a part of the *rpoB* gene, the mechanism of resistance to isoniazid is more complex with mutations conferring resistance in several genomic loci, such as *katG*, *inhA*, *ahpC*, and potentially, *ndh* [5-8]. Mutations in codon 315 of the *katG* gene (*katG*315) and in the promoter region of the *inhA* gene are by far most common. *KatG*315 mutations occur in 50-95% of isoniazid-resistant strains [6, 9, 10] whereas 20-42% of such strains have mutations in the promoter region of the *inhA* gene [6, 10, 11], depending on the geographic region studied.

Isoniazid is activated by the enzyme catalase peroxidase, encoded by *katG* [12]. *KatG* mutations lead to high-level isoniazid resistance (to ≥1.0 μg/ml in 7H10 agar) [13]. The *inhA* gene encodes an enoyl acyl carrier protein reductase involved in fatty acid synthesis. These fatty acids are the target of the active derivative of isoniazid. *InhA* mutations usually lead to low-level isoniazid resistance (resistant to 0.2 μg /ml in 7H10 agar) [13, 14].

*KatG*315 mutations have been shown to be associated with MDR-TB and TB transmission [15, 16]. Such mutations were more frequent among patients infected with Beijing genotype strains [17, 18], which are common in East Asia including Vietnam [19, 20], and related to drug resistance [20, 21], as well as to relapse in various areas [22, 23]. These differences in isoniazid resistance-conferring mutations may also be related to other characteristics of MTB strains, as well as to treatment outcomes. There are, however, very few studies on the mutations underlying resistance to anti-TB drugs and treatment outcome. We therefore studied the epidemiology of *katG* and *inhA* mutations in MTB isolates and the clinical characteristics of the respective patients in Vietnam, where the prevalence of smear-positive TB was 197/100,000 in 2006-2007 [24] and resistance to isoniazid is common (16 to 25% in new patients) [25]. For this we assessed MTB genotype and TB treatment outcomes in association with *katG* and *inhA* mutations in a prospective, population-based study.

Materials and methods

Study subjects

The study area consisted of three adjacent rural districts in Tiengiang Province in the Mekong River Delta in Southern Vietnam. Details of the study have been described elsewhere [26].

From 1 July 2005 to 30 June 2007, all patients aged ≥15 years, resident in the study area and registered for treatment of smear-positive pulmonary TB, were eligible for inclusion after provision of written informed consent. Excluded were patients who received treatment for more than two weeks before registration. Ethical clearance for

the study was obtained from the ethical health committee of the Ho Chi Minh City Council.

According to the guidelines of the Vietnam National TB Control Program [27] patients with no history of treatment with anti-TB drugs for >1 month (i.e. new TB cases) were treated with 2 months of daily streptomycin (S), isoniazid (H), rifampicin (R) and pyrazinamide (Z), followed by daily ethambutol (E) and isoniazid for 6 months (2SHRZ/6EH). Previously treated patients were given all five drugs (SHRZE) daily for 2 months, then four drugs (HRZE) for one more month, followed by RHE 3 days per week for 5 months. All doses were given under directly observed treatment as long as patients were given rifampicin, irrespective of treatment phase or regimen. Drug susceptibility testing was done later, and results were not used to modify treatment regimens. Treatment adherence was confirmed from the treatment cards kept at the district tuberculosis unit (DTU).

Study design

The purpose of the present study was to quantify possible associations between isoniazid resistance-conferring mutations in the MTB strain isolated before treatment and MTB genotype and patient characteristics among all TB patients, and between pre-treatment isoniazid resistance-conferring mutations and treatment outcomes (treatment failure and relapse) among new TB cases. New TB patients were followed up during standard first-line treatment with sputum smear microscopy at months 3, 5 and 8 and with sputum culture at the end of treatment (after 8 months, or after 5 months if the month 5 smear was positive). Participants whose sputum smear and culture were negative for MTB at the end of treatment were visited by study staff twice thereafter, at around 9 and 18 months after treatment completion, or later if not encountered. In addition, data were collected during this period on study participants reporting with TB symptoms at any of the study clinics. Participants who had any complaints suggesting recurrent TB during these visits, or when they themselves consulted a participating clinic, provided two sputum specimens for smear and culture. The data were also collected on any intermediate TB treatment elsewhere, and on causes of death among the study patients based on clinic reports, death certificates and interviews with family members.

Laboratory methods

Sputum specimens were kept refrigerated and transported to Pham Ngoc Thach Hospital in Ho Chi Minh City within 72 h after collection. They were decontaminated and liquefied with 1% N-acetylcystine, 2% NaOH, inoculated on modified Ogawa medium and incubated at 37ºC. Cultures were examined for growth after 1, 2, 4, 6 and 8 weeks of incubation. Cultures with no growth after 8 weeks were reported as negative. MTB was identified using the niacin and the nitrate tests.

Drug susceptibility testing (DST) was performed using the proportion method on Löwenstein-Jensen (LJ) medium [28]. Criteria for drug resistance were ≥1% of the colony forming units grown at 28 or 42 days compared to the drug-free control medium at the following drug concentrations: isoniazid 0.2 μg/ml, rifampicin 40 μg/ml,

streptomycin 4 μg/ml and ethambutol 2 μg/ml [28]. All isoniazid-resistant MTB strains that were isolated were subjected to testing by GenoType® MTBDR*plus* that combines detection of MTB complex with detection of mutations in the 81-bp hotspot region of *rpoB*, at codon 315 of the *katG* gene and in the *inhA* promoter region [15]. All baseline and follow-up isolates from patients with positive follow-up MTB cultures were subjected to molecular typing by spoligo and Variable Number of Tandem Repeats (VNTR) typing. Bacterial DNA was extracted from positive cultures using an earlier described method [29]. Spoligotyping was performed according to the internationally standardized method [30], and VNTR typing was done using 15 loci [31].

Definitions

Previously treated patients were those who received 1 month or more of anti-TB drugs in the past. Cure was defined as a negative sputum smear examination and culture in the last month of treatment and on at least one previous occasion, and treatment failure as any positive sputum smear or culture at 5 months or later during treatment. Treatment completion was defined as having completed treatment without meeting the criteria for being classified as cure or failure.

Recurrent TB was defined as any case of positive smear and/or culture during the follow-up period among the cured patients [32]. We defined a case of recurrent TB as relapse if the initial and follow-up MTB isolates had identical spoligotypes and VNTR patterns, or if the VNTR patterns differed by ≤1 locus, and as reinfection if otherwise [31]. Unfavorable treatment outcome was referred to as treatment failure or death and related to the treatment period only.

Genotypes were based on spoligotyping. The Beijing genotype was defined as any isolate without direct-repeat spacers 1 to 34 and the presence of at least three of the spacers from 35 to 43 [33]. Other genotypes, including the East African-Indian (EAI) genotype that is predominant in Vietnam, were defined as described by Brudey *et al*. [34].

Data analysis

Data were double entered in Epi-Info (version 6.04; Centers for Disease Control and Prevention, Atlanta GA); discrepancies were corrected based on the raw data. Analyses were performed in Stata (version 10SE; Stata Corporation, College Station, TX).

For comparison of categorical variables we used the chi-squared and two-sided Fisher's exact tests as appropriate. Associations of *katG*315 or *inhA* mutations with explanatory variables before start of treatment were expressed as odds ratios; confounding effects were investigated by multivariable logistic regression modeling. In the analysis of treatment failure, mutations in *katG* and *inhA* were assessed by multivariable logistic regression as explanatory variables, along with covariates that showed confounding effects, potentially including age, sex, residence, resistance to other drugs, the MTB genotype, pre-treatment smear grading and extent of chest X-ray abnormalities, and treatment adherence. Only variables that showed confounding effects for the association between resistance mutations and the outcome were retained in the

final model. Since patients who died during treatment may reflect treatment failures, we repeated this analysis taking failure or death as unfavorable treatment outcome. For the association with relapse we did a similar analysis using multivariable Cox' proportional hazard modeling. P values for contribution to multivariate models, including interaction, were based on the likelihood ratio test. All tests were done at the 5% significance level.

Results

After excluding 151 patients (Figure 1), pre-treatment data were available for analysis for 1,213 (88.9%) of 1,364 registered patients. Of these, 924 were male (76.2%); the mean age was 50 years (standard deviation [SD] = 18.3; range 15 to 102). There were 1,102 (90.9%) new patients and 111 (9.1%) patients previously treated for TB.

Of 1,213 *M. tuberculosis* pretreatment isolates, 69 (5.7%) were monoresistant to isoniazid, 146 (12.4%) were monoresistant to streptomycin, 128 (10.6%) were resistant to isoniazid and streptomycin, and 47 (3.9%) were multidrug resistant (see Table S1 in the supplemental material). Monoresistance to isoniazid was more frequent among previously treated patients than among new TB patients (12.6% versus 5.0%, P<0.05), whereas the proportion of other monoresistance patterns did not significantly differ between previously treated and new patients.

Isoniazid resistance-conferring mutations

Of the 251 (20.7%) phenotypically isoniazid resistant MTB strains, 227 (90.4%) exhibited mutations by GenoType® MTBDR*plus* testing; 171 (75.3%) had mutations or no reaction on wild type (WT) probes in *katG*315 including 167 (97.7%) with *katG* S315T1 mutations. Sixty-four (28.2%) had mutations in the *inhA* promoter region, including 61 (95.3%) involving *inhA* C15T mutations. Only 8 of 227 (3.5%) strains with a *katG*315 mutation had an additional mutation in the *inhA* promoter region (Table 1).

Characteristics for* katG315 *mutations

There were no significant associations between the probability of having a strain with a *katG*315 mutation and the patient's district, type of residence, age or presence of mutations in the *inhA* promoter region. However, *katG*315 mutations were significantly more frequent among women (odds ratio [OR] 1.4), among patients previously treated for TB (OR 5.3), among strains that were resistant to rifampicin (OR 27.7), streptomycin (OR 16.9) or ethambutol (OR 62.1), and among strains that belonged to the Beijing genotype (OR 3.2).

Table 1. Results of isoniazid resistance mutations detected by Genotype® MTBDR*plus* test among 227 tuberculosis patients with phenotypic resistance to isoniazid in Vietnam.

Mutation(s)		Frequency	
katG	***inhA***	**No of patients**	**% Total**
WT(315) absent		171	75.3
MUT1 (S315T1)		167	73.6
MUT2(S315T2)		0	0
	WT (-15/-16) absent	56	24.7
	WT (-8) absent	5	2.2
	MUT1(C15T)	61	26.9
	MUT2 (A16G)	0	0
	MUT3A (T8C)	0	0
	MUT3B (T8A)	3	1.3
MUT1 (S315T1)	MUT1(C15T)	8	3.5

In a multivariable model *katG*315 mutations remained associated (adjusted OR [OR^{adj}]; 95% confidence interval [CI]) with previous TB treatment (2.6; 1.4-4.8), resistance to rifampicin (4.5; 1.8-11.1), ethambutol (12.0; 2.7-54.4) or streptomycin (15.0; 9.4-23.9), as well as with female sex (1.9; 1.2-3.0), but not with the Beijing genotype (1.3; 0.8-2.2) or EAI genotype (1.7; 0.9-3.0) compared to all other genotypes together (Table 2). When leaving resistance to other drugs than isoniazid out of the model, the Beijing genotype (2.6; 1.7-4.1), but not the EAI genotype was associated with *katG315* mutation. When adding resistance to only one of the three other drugs to the model, the Beijing genotype was still significantly associated with katG315 mutations after adjustment for rifampicin (2.2; 1.4-3.4) or ethambutol resistance (2.5; 1.6-4.0), whereas this association disappeared after adjustment for streptomycin resistance (1.5; 0.9-2.5).

Table 2. Univariable and multivariable associations with patient characteristics, genotype, and anti-TB drug resistance for *katG* codon 315 mutations at the start of treatment (baseline).

Characteristic or parameter	No of patients	*kat*G mutations		OR (95% CI)[a]		p
		No.	%	Crude	Adjusted	
Total no. of subjects	1213					
Sex						0.009
Male	924	120	13	1	1	
Female	289	51	17.7	1.4 (1.0-2.05)	1.9 (1.2-3.0)	
Age (yrs)						0.349
<25	119	16	13.5	1	1	
25 to 49	528	79	15	1.1 (0.6-2.0)	1.7 (0.8-3.7)	
≥50	566	76	13.4	1.0 (0.6-1.8)	1.7 (0.8-3.7)	
History of tuberculosis						0.002
New	1102	126	11.4	1	1	
Previously treated	111	45	40.5	5.3 (3.4-8.2)	2.6 (1.4-4.8)	
District						
Cailay	440	60	13.6	1		
Caibe	418	57	13.6	1.0 (0.7-1.5)		
Chauthanh	355	54	15.2	1.1 (0.8-1.7)		
Residence						
On waterway	457	67	14.7	1		
On provincial road	624	91	14.6	1.0 (0.7-1.4)		
On national road	132	13	9.9	0.6 (0.3-1.2)		
Genotype family						0.203
East African Indian	461	40	8.7	0.9 (0.6-1.5)	1.7 (0.9-3.0)	
Beijing	407	98	24.1	3.0 (2.0-4.6)	1.3 (0.8-2.2)	
Other	345	33	9.6	1	1	

Table 2, continued. Univariable and multivariable associations with patient characteristics, genotype, and anti-TB drug resistance for *katG* codon 315 mutations at the start of treatment (baseline).

Characteristic or parameter	No. of patients	*kat*G mutations		OR (95% CI)[a]		P
		No.	%	Crude	Adjusted	
Rifampicin resistance						<0.001
No	1163	132	11.4	1	1	
Yes	50	39	78	27.7 (13.1-58.6)	4.5 (1.8-11.1)	
Streptomycin resistance						<0.001
No	889	36	4.1	1	1	
Yes	324	135	41.7	16.9 (10.7-26.7)	15.0 (9.4-23.9)	
Ethambutol resistance						<0.001
No	1184	145	12.3	1	1	
Yes	29	26	89.7	62.1 (17.2-224.4)	12.0 (2.7-54.4)	
***InhA* mutation**						<0.001
No	1149	163	14.2	1	1	
Yes	64	8	12.5	0.9 (0.4-1.9)	0.2 (0.1-0.5)	

[a] "Adjusted" means adjusted for all other variables in the model, CI, Confidence interval;OR, Odds ratio, TB: Tuberculosis.

Table 3. Univariable and multivariable associations with patient characteristics, genotype, and anti-TB drug resistance for the *inhA* promoter region mutations at the start of treatment (baseline).

Characteristic or parameter	No of patients	*inhA* mutations		OR (95% CI)[a]		p
		No.	%	Crude	Adjusted	
Total	1,213					
Sex						0.184
Male	924	46	5.0	1	1	
Female	289	18	6.2	1.3 (0.7-2.2)	1.5 (0.8-2.7)	
Age (yrs)						0.453
<25	119	4	3.4	1	1	
25-<50	528	30	5.7	1.7 (0.6-5.0)	1.9 (0.6-5.9)	
≥50	566	30	5.3	1.6 (0.6-4.7)	1.7 (0.6-5.1)	
History of tuberculosis						0.025
New	1102	51	4.6	1	1	
Previously treated	111	12	11.7	2.7 (1.4-5.2)	2.5 (1.2-5.4)	
District						0.066
Cailay	440	14	3.2	1	1	
Caibe	418	31	7.4	2.4 (1.3-4.7)	2.1 (1.1-4.1)	
Chauthanh	355	19	3.4	1.7 (0.9-3.5)	1.3 (0.6-2.8)	
Residence						
On waterway	457	25	5.5	1		
On provincial road	624	32	5.1	0.9 (0.6-1.6)		
On national road	132	7	5.3	1.0 (0.4-2.3)		
Genotype family						0.276
East African-Indian	461	21	4.6	0.7 (0.4-1.4)	1.0 (0.5-2.0)	
Beijing	407	22	5.4	0.9 (0.5-1.6)	0.6 (0.3-1.2)	
Other	345	21	6.1	1	1	

Table 3, continued. Univariable and multivariable associations with patient characteristics, genotype, and anti-TB drug resistance for the *inhA* promoter region mutations at the start of treatment (baseline).

Characteristic or parameter	No of patients	*inhA* mutations		OR (95% CI)[a]		P
		No.	%	Crude	Adjusted	
Rifampicin resistance						0.496
No	1163	57	4.9	1	1	
Yes	50	7	14.0	3.2 (1.4-7.4)	1.6 (0.5-5.4)	
Streptomycin resistance						<0.001
No	889	31	3.5	1	1	
Yes	324	33	10.2	3.1 (1.9-5.2)	4.4 (2.4-8.1)	
Ethambutol resistance						0.122
No	1184	59	5.0	1	1	
Yes	29	5	17.2	4.0 (1.5-10.8)	3.3 (0.8-14.3)	
***KatG* mutation**						<0.001
No	1042	56	5.4	1	1	
Yes	171	8	4.7	0.9 (0.4-1.8)	0.2 (0.1-0.6)	

[a] "Adjusted" means for all other variables in the model.

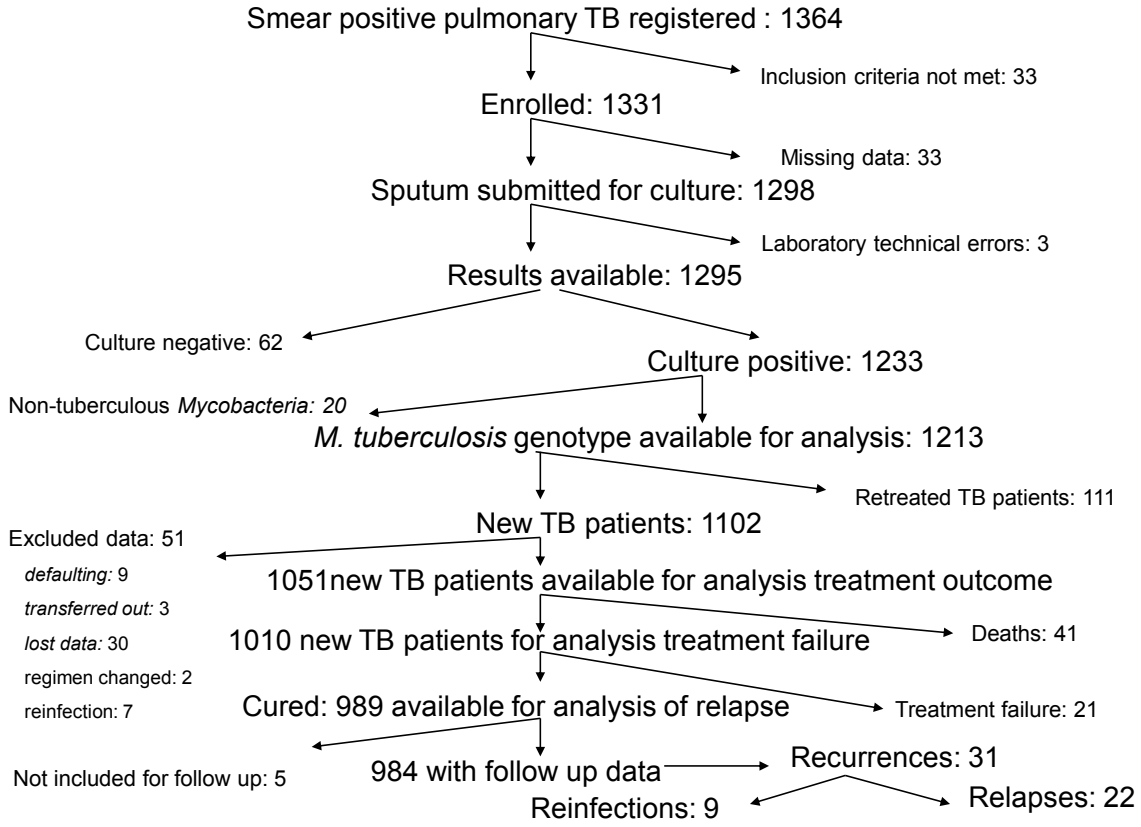

Figure 1. Schematic presentation of enrollment of the study population. TB, tuberculosis.

Characteristics for inhA promoter region mutations

In univariate analysis, *inhA* mutations were associated with previous TB treatment (OR 2.7), with resistance to rifampicin (OR 3.2), streptomycin (OR 3.1) or ethambutol (OR 4.0) and with living in one of the districts (Caibe, OR 2.4), but not with sex, age, residence or genotype. In multivariate analysis also including resistance to other drugs, *inhA* mutations remained significantly associated with resistance to streptomycin (OR_{adj} 4.4; 95%CI 2.4-8.1) and previous TB treatment (OR_{adj} 2.5; 95%CI 1.2-5.4), and near-significantly associated with Caibe district (OR_{adj} 2.1; P = 0.066), but not with resistance to rifampicin or ethambutol (Table 3). There was significant interaction between previous TB treatment and streptomycin resistance: *inhA* mutations were significantly associated with streptomycin resistance among new TB patients (OR_{adj} 6.5, P<0.001) but not among previously treated patients (OR_{adj} 0.36, P>0.05).

Predictors of treatment failure

Of 1,102 new TB patients, 51were excluded due to loss of data (30), re-infection (7), defaulting (9), transfer-out (3) and changed treatment regimen because of side effects (2). Furthermore, we excluded 41 patients who died during treatment, leaving 1,010 new patients for this analysis (Figure 1). Of these 21 (2.1%) had a treatment failure (Table 4).

In univariate analysis, the risk of treatment failure was significantly increased for isoniazid-resistant strains having at least a *katG*315 mutation (OR 13.6; 95%CI 5.3-35.4) but not for isoniazid-resistant strains having *inhA* mutations only (OR 2.9; 95%CI 0.3-14.0) or no mutations (OR 6.9; 95%CI 0.8-59.6). After multivariable adjustment for district and resistance to rifampicin, streptomycin or ethambutol, the association between *katG*315 mutations and treatment failure (OR^{adj}; 95%CI) was no longer significant (3.2; 0.8-12.8; Wald test, p=0.102). Similarly, neither *inhA* mutations only (1.0; 0.1-10.1) nor isoniazid resistance without mutations detectable by the GenoType® MTBDR*plus* assay (1.8; 0.1-24.1) showed significant association with treatment failure (Table 4). There was no significant difference in failure between patients with *katG*315 and patients with *inhA* mutated strains. Isoniazid resistance mutations showed no association with treatment adherence, pretreatment smear grade and extent of pretreatment abnormalities, and none of these variables confounded the observed associations between resistance mutation and treatment failure.

When the 41 patients who had died during treatment were included in the analysis and failure or death (62 of 1051 patients; 5.9%) was combined as unfavorable treatment outcome, its risk was significantly increased for isoniazid-resistant strains having at least a *katG*315 mutation (OR 3.7; 95%CI 2.0-6.7) or no mutations detectable by the GenoType® MTBDR*plus* assay (OR 3.7; 95%CI 1.1-13.3) but not for *inhA* mutations only (OR 1.0; 95%CI 0.2-4.4). In a multivariate model adjusting for covariates that appeared to confound this association (resistance to rifampicin or resistance to streptomycin) unfavorable treatment outcome remained significantly associated with the *katG*315 mutations (OR^{adj} 3.0; 95%CI 1.4-6.8; Wald test P=0.007) but not with no detectable mutations (OR^{adj} 3.4; 95%CI0.9-13.4; P=0.081) (Table 4).

Predictors of relapse

For this analysis, we included all 984 new patients who were smear and culture negative at the end of treatment and available for follow-up (Figure 1). We observed 31 cases of recurrent TB, of which 9 were classified as reinfections and 22 (2.2%) were classified as relapse. There were no relapses among the 17 participants with isoniazid-resistant isolates that did not display any mutation in the GenoType® MTBDR*plus* assay. The three strains that displayed both a *katG*315 and an *inhA* mutation were included in the *katG*315 mutation category.

In univariate analysis, both *katG*315 and *inhA* mutations were strongly associated with relapse; hazard ratios (HR) were 6.7 (95%CI 2.6-16.9) and 8.3 (95%CI 2.6-26.4), respectively (Figure 2). Relapse was also significantly more frequent among participants harboring strains that were of the Beijing genotype (HR 6.2) or streptomycin resistant (HR 4.0), and among those with MDR-TB (HR 7.4).

After multivariable adjustment for genotype and resistance to streptomycin or rifampicin, relapse remained strongly associated with *katG*315 mutations (HR^{adj} 4.3; 95%CI 1.4-13.6, p=0.013) and *inhA* mutations (HR^{adj} 8.7; 95%CI 2.5-30.0, p=0.001) compared against isoniazid-susceptible strains (Table 5). The relapse rate did not differ significantly between strains with *katG*315 mutations and strains with *inhA* mutations. We found no significant interactions.

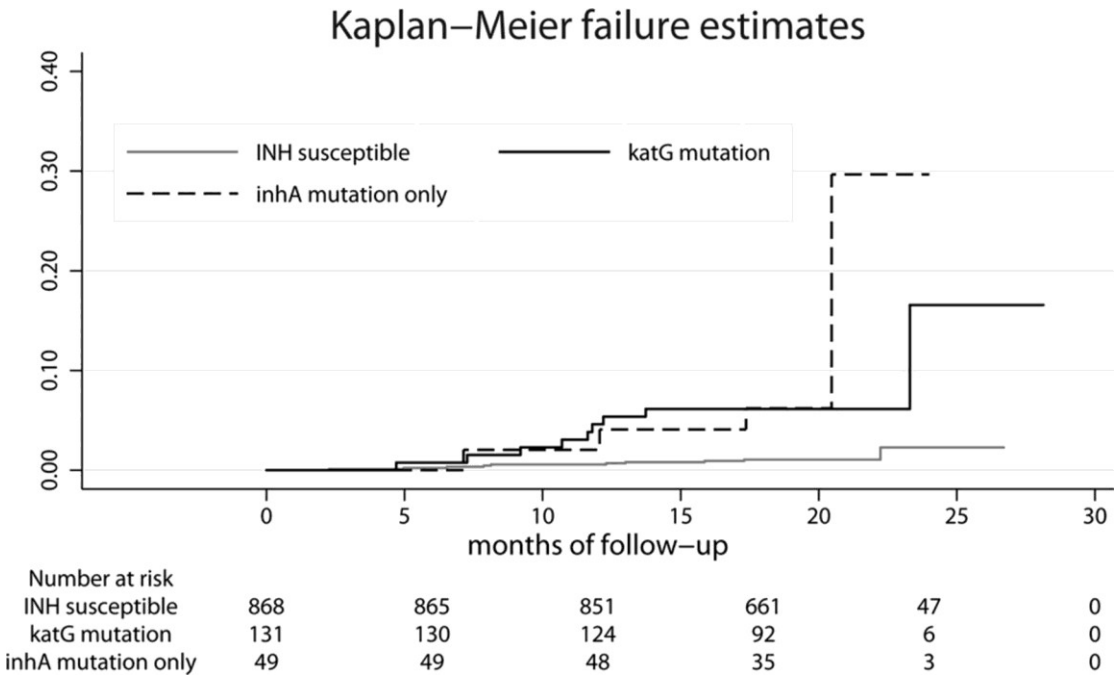

Figure 2. Inverted survival curve for tuberculosis relapse cases among 984 patients after first line TB treatment, isoniazid resistance-conferring mutations. Log-rank test, $P<0.001$. Solid black line, cases due to *katG* codon 315 mutated strains. Interrupted black line, cases due to *inhA* promoter region-only mutated strains. Gray line, cases due to isoniazid-susceptible strains. *y* axis, proportion of relapse cases. INH, isoniazid.

Discussion

In this population-based study conducted in Vietnam, *katG*315 mutations occurred in 75% of the isoniazid-resistant strains and were more often found in strains resistant to rifampicin, streptomycin or ethambutol. In contrast, the *inhA* promoter region mutations were less frequent among isoniazid-resistant strains and only associated with streptomycin resistance. Follow-up of new TB patients on standard first-line treatment showed that *katG*315 and *inhA* promoter region mutations were both strongly associated with relapse. *Kat*G315 mutations showed a 3-fold but non-significant association with treatment failure, while *inhA* promoter region mutations showed no such association at all. Unfavorable treatment outcome was however, significantly associated with *katG*315 mutations, as previously reported by Tolani *et al*. [35]. This was expected because these mutations confer high-level isoniazid resistance [13]. The independent association between *katG315* and failure was non-significant, probably because of small numbers, which is supported by the finding that the association was of similar magnitude but now significant when treatment failure and death were combined. We found no associations between *katG*315 or *inhA* mutations and the Beijing genotype.

Table 4. Multivariable associations for treatment failure or treatment failure and death during treatment combined

	Failure[a]					Failure or death[b]				
Resistance	Total	No.	%	Adjusted OR (95%CI)	P	Total	No.	%	Adjusted OR (95%CI)	P
Isoniazid resistance					0.370					0.046
Susceptible	834	7	0.8	1		866	39	4.5	1	
Any *katG* mutation	116	12	10.3	3.2 (0.8-12.8)	0.102	122	18	14.8	3.0 (1.4-6.8)	0.007
InhA mutations only	42	1	2.4	1.0 (0.1-10.0)	0.974	43	2	4.7	1 (0.2-4.5)	0.994
No mutations	18	1	5.6	1.8 (0.1-24.1)	0.657	20	3	15.0	3.4 (0.9-13.4)	0.081
Rifampicin resistance					0.001					<0.001
No	986	12	1.2	1		1027	53	5.2	1	
Yes	24	9	37.5	7.6 (1.7-34.1)		24	9	37.5	6.3 (2.3-17.1)	
Streptomycin resistance					0.191					0.348
No	760	7	0.9	1		794	41	5.2	1	
Yes	250	14	5.6	2.3 (0.7-8.2)		257	21	8.2	0.7 (0.3-1.5)	
Ethambutol resistance					0.056					
No	997	15	1.5	1		1038	56	5.4	--	
Yes	13	6	46.2	5.9 (1.0-32.3)		13	6	46.2		

[a] The model for the OR and *P* values includes the following covariates: district, isoniazid resistance, rifampin resistance, streptomycin resistance, and ethambutol resistance.

[b] The model for the OR and *P* values includes the following covariates: isoniazid resistance, rifampin resistance, and streptomycin resistance.

Table 5. Multivariable associations for relapse in 967 new tuberculosis patients [a].

	No. of relapses	Incidence per 100 person-years of follow up	Adjusted [b] Hazard ratio	95% CI	*P* value
Total	22				
Isoniazid resistance					0.004
Susceptible	10	0.78	1	--	
Any *katG* mutations	8	5.14	4.3	1.4-13.6	0.013
InhA mutations only	4	6.34	8.7	2.5-30	0.001
Rifampicin resistance	2	9.21	1.2	0.3-5.7	0.821
Streptomycin resistance	12	3.50	1.1	0.4-3.1	0.900
Genotype					<0.001
Beijing	16	3.46	5.1	1.9-14	
Non-Beijing	6	0.58	1	--	

[a] Total of 17 patients with isoniazid-resistant strains for which no mutation was found were excluded from the analysis

[b] Variables in the model: isoniazid resistance mutations, Beijing genotype, streptomycin resistance, rifampicin resistance

A strong association between both *katG*315 and *inhA* mutations and relapse may be caused by the 8-month 2SHRZ/6EH regimen used for new TB patients in Vietnam. This means that not only with high-level but also with low-level isoniazid resistance the supplementation of isoniazid with only ethambutol in the continuation phase of treatment is not effective in sterilizing MTB. Interestingly, the association between isoniazid resistance and relapse was most pronounced for *inhA* mutations. Since the catalase-peroxidase release is a component of the bacterial *OxyR* response, this helps the bacteria to survive inside macrophages [36]. Hence, the probability of survival of bacteria with an *inhA* mutation inside macrophages is higher than for the *katG*315 mutant strains, because they still have full catalase-peroxidase expression. Whether the increased risk of relapse, in particular for *inhA* mutations, also exists with the WHO-recommended 6-month regimen (2RHEZ/4RH), remains to be studied.

In the univariate analysis *katG*315 mutations were strongly associated with the Beijing genotype. Although in accordance with previous studies [9, 17, 18], this association completely disappeared after adjustment for streptomycin resistance. In our study 47% of the Beijing strains were resistant to streptomycin of which 48% also had *katG*315 mutations, suggesting that in the Beijing strains streptomycin resistance and *katG*315 mutations are often present simultaneously. This correlation is not unexpected; streptomycin resistance was also associated with the Beijing genotype, MDR-TB and

increased transmission in the same study area [37]. The association between streptomycin resistance and high-level isoniazid resistance and MDR, especially among Beijing strains, needs further study. It might be related to a specific combination of low-fitness cost mutations conferring these resistances and the strain's genetic background. In addition, yet unknown compensatory mutations may contribute to the strain's fitness.

Our findings also have consequences for the choice of the standard first-line regimen in Vietnam. In line with WHO recommendations, the 8-month regimen for the treatment of new TB patients should be replaced by the 6-month regimen, including rifampicin in the continuation phase.

There were limitations to our study. We only tested isolates for mutations that showed phenotypic resistance to isoniazid and may have missed genotypically isoniazid-resistant isolates. However, drug susceptibility testing was done by an internationally recognized reference laboratory that has consistently shown high concordance rates in proficiency testing therefore this risk is small. We did not determine the minimum inhibitory concentrations of isoniazid for the MTB isolates. We also did not use other genotyping methods to assess the type of mutations conferring isoniazid resistance other than those included in the GenoType® MTBDRplus test. However, the GenoType® MTBDR*plus* test covers ≥ 90% of the isoniazid mutations in MTB isolates in Vietnam, as shown previously [15]. HIV testing was not routinely performed for all patients. However, the HIV infection prevalence in Vietnam is estimated to be 0.4% of the adult population, with substantially lower prevalence in rural provinces than in major cities. We collected no data on clinical characteristics known to predict treatment failure or relapse, such as the presence of co-morbidities or cavities on the chest x-ray. Since it is unlikely that these would be associated with specific isoniazid resistance-conferring mutations before treatment, we do not expect that this resulted in uncontrolled confounding of the observed associations.

In conclusion, isoniazid resistance was most frequently due to mutations in the *katG*315 gene, and these mutations were associated with multidrug and polydrug resistance, whereas *inhA* mutations were less frequent and only associated with streptomycin resistance. Both *katG*315 and *inhA* mutations increased the risk of relapse. Our results also suggest that in Vietnam the 8-month regimen should be discontinued and be replaced by the WHO-recommended 6-month regimen for the treatment new TB patients.

Acknowledgements

This study was supported by KNCV Tuberculosis Foundation, the Netherlands Committee Netherlands-Vietnam and the World Health Organization.

We thank all TB patients participating in this study and the staff of the National Tuberculosis Program of Tiengiang province for recruiting the patients as well as the staff of the National Tuberculosis Program in Pham Ngoc Thach hospital for delivering, supervising and checking the data. We thank Dai Viet Hoa, Phan Thi Hoang Anh, Nhut Kim Phuong, Ho Thi Kim Loan and other staff in Pham Ngoc Thach laboratory for performing culture, DST and GenoType® MTBDR*plus* testing.

We are grateful to Anne-Marie van den Brandt, Mimount Enaimi, Arnout Mulder, Jessica de Beer and other staff of the Tuberculosis Reference Laboratory at the National Institute for Public Health and the Environment (The Netherlands) for providing us the knowledge and practice on the molecular techniques used in this study. We thank Maxine Caws and her staff at the laboratory of the Oxford University Clinical Research Unit (Ho Chi Minh City) for their help in performing the VNTR typing.

References

1. World Health Organization. 2012. Global Tuberculosis Control 2012. Geneva: World Health Organization.
2. Wright A, Zignol M, Van Deun A, Falzon D, Gerdes SR, Feldman K, *et al*. 2009. Epidemiology of antituberculosis drug resistance 2002-07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance. Lancet 373:1861-73.
3. Menzies D, Benedetti A, Paydar A, Royce S, Madhukar P, Burman W, Vernon A, *et al*. 2009. Standardized treatment of active tuberculosis in patients with previous treatment and/or with mono-resistance to isoniazid: a systematic review and meta-analysis. PLoS Med 6:e1000150.
4. Espinal MA, Kim SJ, Suarez PG, Kam KM, Khomenko AG, Migliori GB, *et al*. 2000. Standard short-course chemotherapy for drug-resistant tuberculosis: treatment outcomes in 6 countries. JAMA 283:2537-45.
5. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T, *et al*. 1994. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 263:227-30.
6. Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D, van Embden JD. 1996. Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. J Infect Dis 173:196-02.
7. Kelley CL, Rouse DA, Morris SL. 1997. Analysis of ahpC gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 41:2057-8.
8. Lee AS, Teo AS, Wong SY. 2001. Novel mutations in ndh in isoniazid-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 45:2157-9.
9. Mokrousov I, Narvskaya O, Otten T, Limeschenko E, Steklova L, Vyshnevskiy B. 2002. High prevalence of *KatG* Ser315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from northwestern Russia, 1996 to 2001. Antimicrob Agents Chemother 46:1417-24.
10. Telenti A, Honore N, Bernasconi C, March J, Ortega A, Heym B, *et al*. 1997. Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. J Clin Microbiol 35:719-23.
11. Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. 2008. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am J Respir Crit Care Med 177:787-92.
12. Zhang Y, Heym B, Allen B, Young D, Cole S. 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 358:591-3.

13. Ando H, Kondo Y, Suetake T, Toyota E, Kato S, Mori T, Kirikae T. 2010. Identification of *katG* mutations associated with high-level isoniazid resistance in *Mycobacterium tuberculosis.* Antimicrob Agents Chemother 54:1793-9.
14. Dalla Costa ER, Ribeiro MO, Silva MS, Arnold LS, Rostirolla DC, Cafrune PI, *et al*. 2009. Correlations of mutations in *katG*, *oxyR-ahpC* and *inhA* genes and in vitro susceptibility in *Mycobacterium tuberculosis* clinical strains segregated by spoligotype families from tuberculosis prevalent countries in South America. BMC Microbiol 9:39.
15. Huyen MN, Tiemersma EW, Lan NT, Cobelens FG, Dung NH, Sy DN, *et al*. 2010. Validation of the GenoType MTBDR*plus* assay for diagnosis of multidrug resistant tuberculosis in South Vietnam. BMC Infect Dis 10:149.
16. van Doorn HR, de Haas PE, Kremer K, Vandenbroucke-Grauls CM, Borgdorff MW, van Soolingen D. 2006. Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of *katG*: a decade of experience in The Netherlands. Clin Microbiol Infect 12:769-75.
17. Hillemann D, Kubica T, Rusch-Gerdes S, Niemann S. 2005. Disequilibrium in distribution of resistance mutations among *Mycobacterium tuberculosis* Beijing and non-Beijing strains isolated from patients in Germany. Antimicrob Agents Chemother 49:1229-31.
18. Ghebremichael S, Groenheit R, Pennhag A, Koivula T, Andersson E, Bruchfeld J, *et al*. 2010. Drug resistant *Mycobacterium tuberculosis* of the Beijing genotype does not spread in Sweden. PloS One 5:e10893.
19. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K, *et al*. 2000. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis 6:302-5.
20. Glynn JR, Kremer K, BorgdorffMW, Rodriguez MP, Soolingen DV. 2006. Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. Emerg Infect Dis 12:736-743.
21. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, *et al*. 2009. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis 13:900-6.
22. Burman WJ, Bliven EE, Cowan L, Bozeman L, Nahid P, Diem L, Vernon A. 2009. Relapse associated with active disease caused by Beijing strain of *Mycobacterium tuberculosis*. Emerg Infect Dis 15:1061-7.
23. Huyen MN, Buu TN, Tiemersma E, Lan NT, Dung NH, Kremer K, *et al*. 2013. Tuberculosis relapse in Vietnam is significantly associated with *Mycobacterium tuberculosis* Beijing genotype infections. J Infect Dis 207:1516-24.
24. Hoa NB, Sy DN, Nhung NV, Tiemersma EW, Borgdorff MW, Cobelens FG. 2010. National survey of tuberculosis prevalence in Vietnam. Bull World Health Organ 88:273-80.
25. Huong NT, Lan NT, Cobelens FG, Duong BD, Co NV, Bosman MC, *et al*. 2006. Antituberculosis drug resistance in the south of Vietnam: prevalence and trends. J Infect Dis 194:1226-32.
26. Buu TN, Huyen MN, van Soolingen D, Lan NT, Quy HT, Tiemersma EW, *et al*. 2010. The *Mycobacterium tuberculosis* Beijing genotype does not affect tuberculosis treatment failure in Vietnam. Clin Infect Dis 51:879-86.
27. Huong NT, Duong BD, Co NV, Quy HT, Tung LB, Bosman M, *et al*. 2005. Establishment and development of the National Tuberculosis Control Programme in Vietnam. Int J Tuberc Lung Dis 9:151-6.

28. World Health Organization. 2003. Guidelines for surveillance of drug resistance in tuberculosis. 2nd ed. WHO/CDS/TB/2003.320. Geneva: World Health Organization.
29. van Soolingen D, de Haas PEW, Kremer K. 2001. Restriction fragment length polymorphism typing of mycobacteria, p. 165-203. *In* T. Parish, Stoker NG (ed.), In: *Mycobacterium tuberculosis* protocols. Humana Press Inc., Totowa, New Jersey, USA.
30. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, *et al*. 1997. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 35:907-14.
31. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, *et al*. 2006. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 44:4498-510.
32. World Health Organization. 2009. Treatment of tuberculosis: guidelines. 4th ed. WHO/HTM/TB/2009.420. Geneva: World Health Organization.
33. Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN, *et al*. 2004. Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. J Clin Microbiol 42:4040-9.
34. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, *et al*. 2006. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol 6:23.
35. Tolani MP, D'Souza D T, Mistry NF. 2012. Drug resistance mutations and heteroresistance detected using the GenoType MTBDR*plus* assay and their implication for treatment outcomes in patients from Mumbai, India. BMC Infect Dis 12:9.
36. Farr SB, Kogoma T. 1991. Oxidative stress responses in Escherichia coli and Salmonella typhimurium. Microbiol Rev 55:561-85.
37. Buu TN, van Soolingen D, Huyen MN, Lan NT, Quy HT, Tiemersma EW, *et al*. 2012. Increased transmission of *Mycobacterium tuberculosis* Beijing genotype strains associated with resistance to streptomycin: a population-based study. PloS One 7:e42323.

CHAPTER 7

Characterization of *Mycobacterium tuberculosis* isolates lacking IS*6110* in Vietnam

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International Journal of Tuberculosis and Lung Disease, 2013, 17(11): 1479-1485.

Abstract

Background
The molecular diagnosis of tuberculosis (TB) in Vietnam is often based on the detection of insertion sequence IS*6110* in *Mycobacterium tuberculosis*. However, 8-11% of *M. tuberculosis* strains in South-East Asia do not contain this target and this undermines the validity of these molecular tests.

Methods
We quantified the frequency of *M. tuberculosis* strains lacking IS*6110* in rural Vietnam and studied their epidemiological and clinical characteristics.
Consecutively diagnosed adult TB patients in rural Southern Vietnam submitted two sputum samples for culture, IS*6110* restriction fragment length polymorphism (RFLP), spoligotyping and 15-loci variable number tandem repeat (VNTR) typing. Polymerase chain reaction (PCR) was performed to confirm the absence of the IS*6110* elements in strains lacking IS*6110* hybridization in RFLP.

Results
Among 2,664 TB patient isolates examined, 109 (4.1%) had no IS*6110* element. Compared to other strains, these no-copy strains were less often resistant to anti-tuberculosis drugs, particularly to streptomycin (adjusted odds ratio 0.2, 95% confidence interval 0.1-0.5) and showed significant geographic variation. No associations with TB history or demographic factors were found.

Conclusions
Strains without the IS*6110* target pose a problem in Vietnam as regards false-negative molecular TB diagnosis in PCR. Compared to other strains circulating in Vietnam, no-copy strains are more susceptible to anti-tuberculosis drugs.

Introduction

Tuberculosis (TB) remains one of the most important infectious diseases worldwide. In 2011, 8.7 million TB cases were notified by the World Health Organization (WHO) and approximately 1.4 million people died of the disease [1]. The vast majority of TB cases are still diagnosed using acid-fast bacilli smear microcopy [2], which, due to its low sensitivity, means that many TB patients are not diagnosed in time and continue to transmit TB in the community.

New approaches in the diagnosis of TB are being explored, including polymerase chain reaction (PCR), in which a specific fragment of DNA in the causative agent, *Mycobacterium tuberculosis*, is targeted and amplified to a detectable level. PCR is rapid, sensitive and specific, and can be used for the rapid diagnosis of both pulmonary [3] and extra-pulmonary TB [4]. The insertion sequence (IS) *6110* element is one of the most widely used targets, due to its omnipresence in *M. tuberculosis* complex strains and its absence in non-tuberculous mycobacteria [5,6]. However, the number of IS*6110* copies varies significantly by strain, from 0 to 25, and the consequences of this variation as regards fluctuations in sensitivity have not yet been explored. *M. tuberculosis* strains that lack an IS*6110* element (no-copy strains) exist, as was already described in 1993 [7] soon after the introduction of IS*6110* restriction fragment length polymorphism (RFLP) typing for an isolate from an Indian patient, and the existence of such no-copy strains in Vietnam was confirmed as early as 1995 [8]. Using RFLP typing, one study in India reported a proportion of no-copy strains of 11% [9], whereas in the United States such strains are extremely rare [10].

The diagnostic laboratory test routinely used to diagnose TB in Vietnam is still Ziehl-Neelsen (ZN) microscopy, whereas IS*6110* PCR is often used for ZN negative cases with a high suspicion of TB or in the private sector for early diagnosis of TB. There are diagnostic molecular assays that do not target IS*6110*, such as the Amplified MTD® test (Gen-Probe, San Diego, CA, USA) and the GenoType® MTBDR*plus* test (Hain Lifescience, Nehren, Germany); however, these are much more expensive than the locally produced commercial IS*6110* PCR that is used in most Vietnamese laboratories. In Vietnam, strains without IS*6110* DNA may therefore lead to significant diagnosis of false-negative TB among smear-negative patients.

Most studies on biological variations between the different phylogenetic lineages have focused on strains of the Beijing genotype, which is highly prevalent in (South) East Asian countries such as China and Vietnam [11], and only a few studies have investigated no-copy strains [9,10]. It was recently estimated that no fewer than 8% of the *M. tuberculosis* strains in Vietnam lack IS*6110* DNA (unpublished data), but their molecular characteristics and their clinical and epidemiological characteristics remain unclear.

We performed a population-based prospective study on TB transmission dynamics in rural Vietnam, which gave us the opportunity to determine the frequency of no-copy strains more accurately. We also studied the molecular characteristics of these no-copy strains and determined their association with patient characteristics, spread and drug resistance patterns.

Material and methods

Study population

A population-representative sample of smear-positive sputum specimens was collected from TB patients in three adjacent rural districts (Cailay, Caibe, Chauthanh) in Tien Giang Province, situated in the Mekong River Delta in Southern Vietnam. Molecular typing was performed at the Pham Ngoc Thach Hospital in Ho Chi Minh City, Vietnam.

All patients aged ≥15 years, resident in the study area and registered for treatment of smear-positive pulmonary TB between 1 January 2003 and 28 June 2007 at the participating district TB units or at the provincial TB hospital were eligible for inclusion in the study. Confirmation of the TB diagnosis relied on examination of at least two ZN-stained sputum smears [12]. Eligible patients were included after providing written informed consent; each submitted two sputum samples. For each patient sex, age, bacilles Calmette-Guerine (BCG) vaccination status, education, marital status, profession and previous history of anti-tuberculosis treatment were recorded using a standard questionnaire. Human immunodeficiency virus testing was not routinely performed. Patients who had been on treatment for TB for more than two weeks for this disease episode were excluded.

This study was approved by the ethical health committee of the Ho Chi Minh City Council.

Mycobacterium tuberculosis culture

Sputum specimens were kept refrigerated and transported to Pham Ngoc Thach hospital in Ho Chi Minh City within 72 h. They were decontaminated and liquefied with 1% N-acetylcystine, 2% sodium hydroxide, inoculated on modified Ogawa medium and incubated at 37ºC. Cultures were examined for growth after 1, 2, 4, 6 and 8 weeks of incubation. Cultures with no growth after 8 weeks were reported as negative. *M. tuberculosis* complex was identified using the niacin and the nitrate tests [13].

Drug susceptibility testing

Drug susceptibility testing was performed by the proportion method per WHO guidelines. Criteria for drug resistance were ≥1% colony growth at 28 or 42 days on Löwenstein-Jensen (LJ) medium compared to drug-free control LJ medium, at the following drug concentrations: isoniazid (INH) 0.2 μg/ml, rifampicin (RMP) 40 μg /ml, streptomycin (SM) 4 μg /ml and ethambutol (EMB) 2 μg /ml [14].

DNA typing

Genomic DNA was extracted from positive cultures by a method described elsewhere [15]. IS*6110* RFLP and spoligotyping were performed as per internationally standardized methods [15-17]. Variable number tandem repeat (VNTR) typing was performed on the basis of 15 loci, as described by Supply *et al.* [18]. To determine the presence of IS*6110* elements in the *M. tuberculosis* genome, the template DNA was

targeted by specific IS*6110* primers on PCR, and PCR products were checked on 1.5% agarose gel [15].

Selection of the no IS*6110 *copy strains

In 2004, we reported that more than 30% of *M. tuberculosis* in this area were East African-Indian (EAI) strains, EAI 4VNM and EAI 5, which often harbour fewer than five IS*6110* copies [19]. RFLP typing was thus unable to discriminate between such low-copy strains, and we replaced RFLP by VNTR typing for all strains. As RFLP analysis was conducted for only a subset of the isolates in this study, we used similarity on the basis of spoligotyping results to trace other potential no-copy strains among these strains without RFLP results. A spoligo prototype of the "zero copy" clade suggested in 2006 by Brudey *et al.* has the spoligo international type (SIT n°) 405 [20]. Spoligo patterns of all isolates were compared using a dendrogram. Isolates that showed ≥ 90% similarity with the spoligo patterns of the no-copy strains detected using RFLP typing in the dendrogram were subjected to IS*6110* PCR. If this PCR did not yield a PCR product, the strains were considered no-copy strains.

No-copy strains were defined as *M. tuberculosis* complex strains with no bands in IS*6110* RFLP typing or with a spoligo pattern characteristic of no-copy strains and no product in the IS*6110* PCR.

Data management and analysis

Data were double-entered in Epi Info, version 6.04 (Centers for Disease Control and Prevention, Atlanta, GA, USA); discrepancies were corrected based on the raw data. Bionumerics software, version 3.0 (Applied Maths, Sint-Martens Latem, Belgium) was used for the analysis and comparison of IS*6110* RFLP, spoligo and VNTR patterns.

Statistical analyses were performed in Epi Info 6.04 and Stata 10SE (Stata Corp., College Station, TX, USA). For comparison of categorical variables, we used the chi-squared and 2-sided Fisher's exact tests, as appropriate. Associations with explanatory variables were expressed as odds ratios (ORs); confounding effects were investigated by multivariable logistic regression modeling. All tests were performed at the 5% significance level. In a secondary analysis, we excluded from the comparator all Beijing strains, as these may have specific characteristics, including associations with resistance.

Results

During the study period 2,851 smear-positive TB patients were registered, of whom 2,664 (93.4%) were available for analysis, including 1,996 (74.9%) males and 668 (25.1%) females, with a median age of 49 years (25^{th} and 75^{th} percentile 36 and 65.5 years, respectively). Of these, 2,386 (89.6%) were new patients, 255 (9.6%) had been previously treated and the remaining 23 (0.8%) were of unknown status. Among 2,664 isolates with spoligotyping results, 2,526 (94.8%) were successfully subjected to VNTR typing and yielded a complete 15-loci pattern, and 1,797 (67.5%) were subjected to RFLP typing. Based on our definition for the selection of no IS*6110* copy strains, 60 no-copy strains were identified directly using RFLP. For 59 potential no-copy strains, no

RFLP pattern was available. These strains were selected because their spoligo patterns showed ≥ 90% similarity to strains known to lack IS*6110* using RFLP typing. In total, 109 of 2664 (4.1%) strains were confirmed as lacking any IS*6110* element on IS*6110* PCR (Figure 1).

Of 109 no-copy strains, 71 (65.1%) were missingspacers 19 to 41, and 13 (11.9%) strains were missing spacers 19 to 25 and 27 to 41. The remaining strains missed other spacers, but were observed less frequently (<6%; Figure 2) [21]. Of 109 no-copy strains, 20 (18.3%) had the VNTR pattern 642253245272461 often seen in EAI but not in Beijing strains; the remaining VNTR patterns were observed in <10% of the isolates (Table 1).

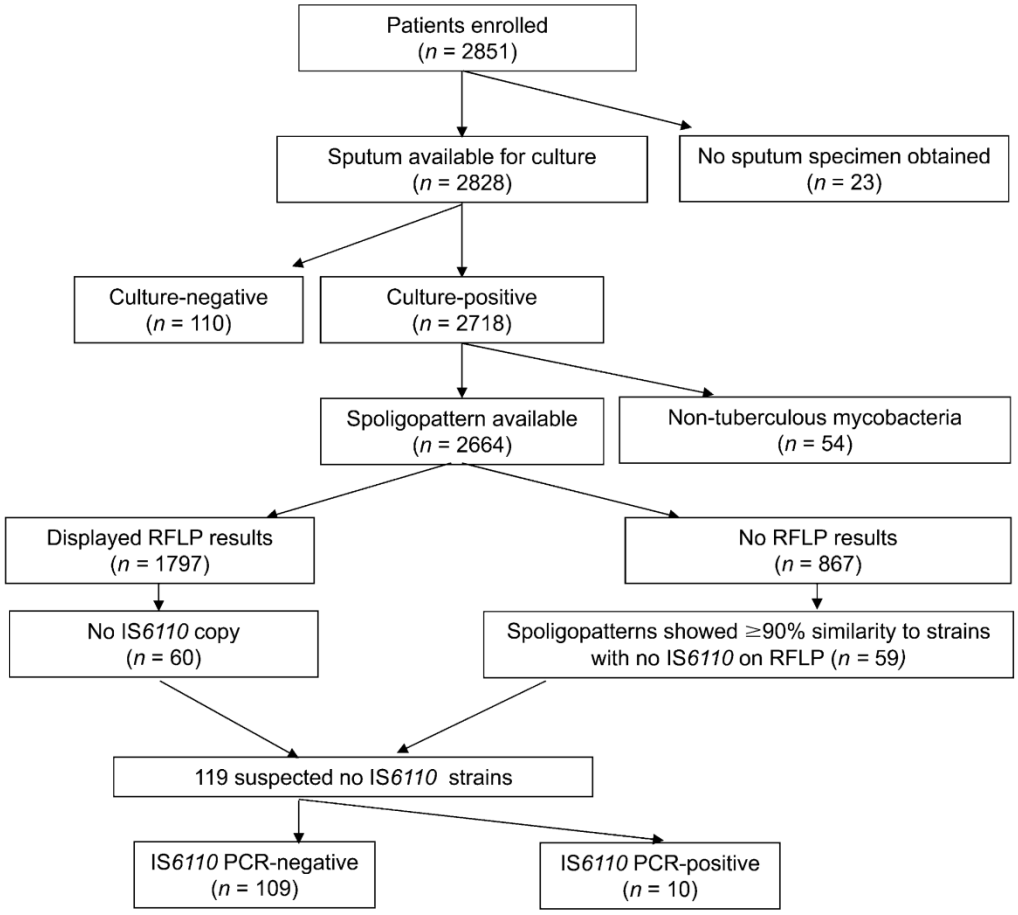

Figure 1. Study flow chart. The data analysis is based on isolates for which a spoligo pattern was available. RFLP = restriction fragment length polymorphism; IS = insertion sequence; PCR = polymerase chain reaction.

Association between no-copy signature and epidemiological factors and drug resistance

In univariate analysis, there were no significant associations between the IS*6110* no-copy signature and sex, age or year of inclusion of the patient nor with susceptibility of the isolate to EMB. A no-copy signature was significantly associated with susceptibility to INH (P=0.003) and SM (P<0.001), and was significantly more frequent

in one of the three districts (Chauthanh; P=0.027). In a multivariable model including the variables districts, sex, age, history of TB, INH resistance and SM resistance, a no-copy signature remained significantly associated with susceptibility to SM (adjusted OR [aOR] 0.2, 95%CI 0.1-0.5) and Chauthanh district (aOR 2.0, 95%CI 1.2-.3.1) (Table 2).

Compared to all strains except those belonging to the Beijing genotype, no-copy signatures were significantly associated with susceptibility to SM (aOR for resistance versus susceptibility to SM 0.4, 95%CI 0.2-0.9) and being isolated from Chauthanh district (data not shown).

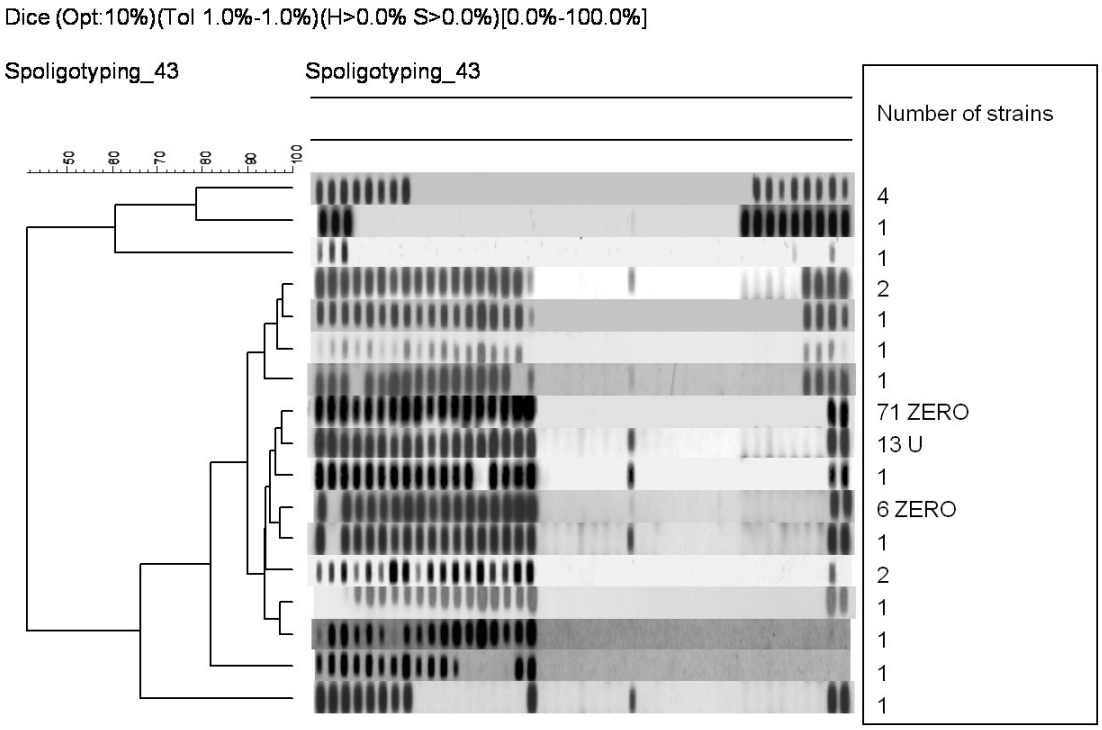

Figure 2. Spoligotyping patterns of the 109 no-copy *M. tuberculosis* strains identified in this study. The figure shows the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) dendrogram (left) that was constructed based on the similarity of the spoligo patterns, the spoligo patterns (middle) and the number of strains showing similar spoligo pattern results (right). Among the 109 no-copy strains, 17 different spoligo patterns were identified. A spacer showing weak signal was considered to be absent when its intensity was 15% or less of the average intensities of the other bands in the same lane [21].

Discussion

In this population-based study among TB patients in a rural area in Southern Vietnam, we found that no-copy IS*6110* strains are relatively frequent, with a prevalence of 4.1%. No-copy signatures were associated with susceptibility to anti-tuberculosis drugs, especially SM. These associations are in contradiction with those found earlier

for the Beijing genotype family, which was significantly associated both with resistance to anti-tuberculosis drugs, especially SM and INH [22], and with multidrug resistant TB [23-25]. The analysis yielded similar results after excluding Beijing strains.

Table 1. Frequency of 66 no-copy strains sharing VNTR patterns between *M. tuberculosis* families *

VNTR patterns often shared by strains	No-copy Strains (n=109) n (%)	East-African Indian strains (n=917) n (%)	Other genotype Strains (n=580) n (%)	Beijing strains (n=910) N
642253245272461	20 (18.3)	101 (11)[¥]	28 (4.7)	0
642253245272421	10 (9.2)	1 (0.1)	0	0
642253245262461	9 (8.3)	7 (0.8)	3 (0.5)	0
542253245272461	6 (5.5)	8 (0.9)	6 (1)	0
642253245272261	5 (4.6)	1 (0.1)	1 (0.2)	0
542251245272461	3 (2.8)	2 (0.2)	2 (0.3)	0
742253245272461	1 (0.9)	7 (0.8)	1 (0.2)	0
642253246272461	1 (0.9)	1 (0.1)	0	0
642252245272461	1 (0.9)	1 (0.1)	17 (2.9)	0
642252245272451	1 (0.9)	2 (0.2)	2 (0.3)	0
542253245262461	1 (0.9)	2 (0.2)	1 (0.2)	0
542253245272561	1 (0.9)	1 (0.1)	1 (0.2)	0
642253245172461	1 (0.9)	4 (0.4)	0	0
642253245262561	1 (0.9)	2 (0.2)	0	0
632253245272461	1 (0.9)	23 (2.5)	8 (1.4)	0
642253245252461	1 (0.9)	3 (0.3)	2 (0.3)	0
642253245272661	1 (0.9)	1 (0.1)	0	0
642253245282461	1 (0.9)	1 (0.1)	0	0
642253245272411	1 (0.9)	1 (0.1)	0	0

*43 no-copy strains with different VNTR patterns are not mentioned in this table. *Mycobacterium* families were confirmed based on SpolDB4 [20].
[¥]The most frequent common VNTR pattern between the no-copy and the EAI.
VNTR = variable number tandem repeat; EAI = East- African- Indian.

As these strains have no copy of the IS*6110* element in their genome, which is used as a target sequence in the IS*6110* PCR, they cannot be detected by this test. To date, at least six laboratories in the South Vietnam have used the IS*6110*-based PCR to diagnose TB; this implies that a significant number of smear-negative TB patients may currently be misdiagnosed by PCR in this area and may remain a source of TB transmission in the community. IS*6110* PCR should thus be replaced by another PCR method does not use IS*6110* as a target, such as, for example, a specific *rpoB* gene signature, which is used

in GeneXpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA)[26] and is present in all *M. tuberculosis* complex strains, or by IS*1081,* which is usually present in multiple copies in strains that lack IS*6110* [7,27].

The frequency of no-copy strains (4.1%) in our study was significantly lower (P <0.001) than in previous studies in Vietnam and India, both of which detected no-copy strains using RFLP only, and in which the frequency of these strains was estimated at respectively 8% and 11% of tested isolates (N.T.N. Lan, personal communication) [9]. This difference is probably due not only to the differences in geographical distribution of genotypes of *M. tuberculosis*, but also to the identification and verification of no-copy strains. We used RFLP and spoligotyping to screen for IS*6110*-devoid strains and used PCR to confirm IS*6110* PCR for verification. It is conceivable that this approach has a higher specificity for detecting no-copy strains, resulting in fewer misclassifications of strains with IS*6110* bands as no-copy strains than when only RFLP typing is performed. Lok *et al*. found that no-copy strains were extremely rare in the United States (0.18%) and that these were usually found in persons originating from South-East Asia [10].

A potential explanation as to why South-East Asians are more frequently infected with no-copy strains than, for example, Americans of other origins may be the historical geographical isolation of South-East Asian populations, or an as yet unknown co-evolution of host and pathogen. No-copy strains are represented among the EAI lineage (as defined by spoligotyping, Brudey *et al*. [20], also known as Lineage 1 or Rim of Indian Ocean, as defined by single nucleotide polymorphisms [SNPs]) [28,29]. Based on SNPs, no-copy strains represent the "ancient lineage", a lineage that has more similarity to the common ancestor of all *M. tuberculosis* complex strains than strains of the "modern lineage", which have undergone more mutations. Strains of the Beijing genotype, which, in contrast to the no-copy strains, show a strong association with anti-tuberculosis drug resistance, belong to the modern lineage. The acquired mutations may have given the Beijing genotype strains an advantage in their adaptation to the pressure of BCG vaccination [30] and treatment by anti-tuberculosis drugs. Moreover, in a recent study by De Steenwinkel *et al*., it was shown that a part of the Beijing genotype strains have much higher intrinsic resistance to treatment by RMP, as well as a higher mutation frequency in the generation of RMP-resistant mutants in comparison with EAI isolates [31].

This most likely indicates that no-copy strains are, in terms of evolution, less fit to deal withthe current measures against TB; they may therefore represent an interesting group of strains for studying the evolutionary development and adaptation of *M. tuberculosis* to the current measures against TB.

There are some limitations in our study. First, we did not use other markers such as TbD1 to test no-copy strains. Second, RFLP typing was not applied to screen for IS*6110* in all isolates; however, all isolates were screened using spoligotyping, and the results were confirmed using IS*6110* PCR. Finally, this study included only patients from a limited area in the south of Vietnam, and it is not known if the findings can be extrapolated to other regions in this country.

TABLE 2. Uni- and multivariable associations between no-copy strains and epidemiological characteristics and drug resistance.

Characteristics	Total	Patients n (%)	Crude OR	P value	Adjusted OR*	95% CI	P value
Age (yrs)				0.278			0.265
15-34	563	18 (3.2)	1		1		
35-64	1397	56 (4)	1.3		1.3	0.8-2.3	
>= 65	704	35 (5)	1.6		1.6	0.9-2.9	
Sex				0.880			0.790
Male	1996	81 (4.1)	1		1		
Female	668	28 (4.2)	1.03		1.06	0.7-1.7	
District				0.027			0.015
Cailay	1084	33 (3.0)	1		1		
Caibe	784	32 (4.1)	1.4		1.3	0.8-2.1	
Chauthanh	796	44 (5.5)	1.9		2.0	1.2-3.1	
TB treatment history				0.051			0.141
New patient	2386	105 (4.4)	1		1		
Previously treated patient	255	4 (1.6)	0.4		0.5	0.2-1.3	
Unknown	23	0					
Isoniazid resistance				0.003			0.726
Yes	543	10 (1.8)	0.4		0.9	0.4-1.8	
No	2121	99 (4.7)	1		1		
Rifampicin resistance				0.061			
Yes	126	1 (0.8)	0.18				
No	2538	108 (4.3)	1				
Streptomycin resistance				<0.001			<0.001
Yes	743	8 (1.1)	0.1		0.2	0.1-0.5	
No	1921	101 (5.3)	1		1		

Table 2, continued. Uni- and multivariable associations between no-copy strains and epidemiological characteristics and drug resistance.

Characteristics	Total	Patients n (%)	Crude OR	P value	Adjusted OR*	95% CI	P value
Ethambutol resistance				0.479			
Yes	48	1 (2.1)	0.49				
No	2616	108 (4.1)	1				
Multidrug resistance				0.09			
Yes	108	1 (0.9)	0.2				
No	2556	108 (4.2)	1				

* Adjustment for districts, sex, age, history of TB, and isoniazid and streptomycin resistance.
OR = odds ratio; CI = confidence interval; TB = tuberculosis.

Conclusions

The frequency of strains without IS*6110* DNA in a rural part of South Vietnam was 4.1%. This suggests that TB is underdiagnosed in Vietnam, as PCR tests targeting IS*6110* are commonly used in Vietnam. Compared to other strains, drug resistance, especially to SM, occurred at lower frequencies in the no-copy family.

Acknowledgements

The authors thank all TB patients participating in this study and the staff of the National Tuberculosis Program (NTP) of Tien Giang province for recruiting patients, as well as the staff of the NTP of Pham Ngoc Thach hospital for supervision and data verification. Dai Viet Hoa, Phan Thi Hoang Anh, and other staff at the Pham Ngoc Thach laboratory are thanked for performing culture, spoligotyping, RFLP and VNTR typing. The authors are grateful to Anne-Marie van den Brandt, Arnout Mulder, Mirjam Dessens, Mimount Enaimi and Jessica de Beer and other staff of the Tuberculosis Reference Laboratory at RIVM, The Netherlands, for providing their knowledge and expertise in the molecular techniques used in this study. They also thank Maxine Caws and her staff at the laboratory of the Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam, for their help in performing VNTR typing.

This research was funded by the KNCV Tuberculosis Foundation and the Medical Committee Netherlands-Vietnam, The Netherlands.

References

1. World Health Organization. Global tuberculosis control report 2012. WHO/HTM/TB/2012.6. Geneva: WHO, 2012.
2. Das S, Paramasivan CN, Lowrie DB, Prabhakar R, Narayanan PR. IS*6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, south India. Tubercle Lung Dis. 1995; 76: 550-4.
3. El-Dawi TG, Saeed el NS, Hamid ME. Evaluation of a PCR-amplified IS*6110* insertion element in the rapid diagnosis of pulmonary tuberculosis in comparison to microscopic methods in Sudan. Saudi Med J. 2004; 25: 1644-7.
4. Deshpande PS, Kashyap RS, Ramteke SS, Nagdev KJ, Purohit HJ, Taori GM, *et al*. Evaluation of the IS*6110* PCR assay for the rapid diagnosis of tuberculous meningitis. Cerebrospin Fluid Res. 2007; 4:10.
5. Hellyer TJ, DesJardin LE, Assaf MK, Bates JH, Cave MD, Eisenach KD. Specificity of IS*6110*-based amplification assays for *Mycobacterium tuberculosis* complex. J Clin Microbiol. 1996; 34: 2843-6.
6. Marchetti G, Gori A, Catozzi L, Vago L, Nebuloni M, Rossi MC, *et al*. Evaluation of PCR in detection of *Mycobacterium tuberculosis* from formalin-fixed, paraffin-embedded tissues: comparison of four amplification assays. J Clin Microbiol. 1998; 36: 1512-7.
7. van Soolingen D, de Haas PE, Hermans PW, Groenen PM, van Embden JD. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. J Clin Microbiol. 1993; 31: 1987-95.
8. Thierry D, Chavarot P, Marchal G, Le Thi KT, Ho ML, Nguyen NL, *et al*. *Mycobacterium tuberculosis* strains unidentified using the IS*6110* probe can be detected by oligonucleotides derived from the Mt308 sequence. Res Microbiol. 1995; 146: 325-8.

9. Chauhan DS, Sharma VD, Parashar D, Chauhan A, Singh D, Singh HB, *et al*. Molecular typing of *Mycobacterium tuberculosis* isolates from different parts of India based on IS*6110* element polymorphism using RFLP analysis. Indian J Med Res. 2007; 125: 577-81.
10. Lok KH, Benjamin WH, Jr., Kimerling ME, Pruitt V, Lathan M, Razeq J, *et al*. Molecular differentiation of *Mycobacterium tuberculosis* strains without IS*6110* insertions. Emerg Infect Dis. 2002; 8: 1310-3.
11. Glynn JR, Kremer K, BorgdorffMW, Rodriguez MP, Soolingen DV. Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. Emerg Infect Dis. 2006; 12: 736-43.
12. World Health Organization. Treatment of tuberculosis. Guidelines for national programmes. 3rd ed. WHO/CDS/TB/2003.313. Geneva: WHO, 2003.
13. International Union Against Tuberculosis and Lung Disease. Technical guide-Sputum examination for tuberculosis direct microscopy in low-income countries. 5th ed. Paris: The Union, 2000.
14. World Health Organization. Guidelines for surveillance of drug resistance in tuberculosis.2nd ed. WHO/CDS/TB/2003.320. Geneva: WHO, 2003.
15. van Soolingen D, de Haas PEW, Kremer K. Restriction fragment length polymorphism typing of mycobacteria. In: T. Parish, Stoker NG, editors. In: *Mycobacterium tuberculosis* protocols. Totowa, New Jersey: Humana Press Inc.; 2001. p. 165-203.
16. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, *et al*. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol. 1993; 31: 406-9.
17. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, *et al*. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 1997; 35: 907-914.
18. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, *et al*. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol. 2006; 44: 4498-510.
19. Buu TN, van Soolingen D, Huyen MN, Lan NT, Quy HT, Tiemersma EW, *et al*. Increased transmission of *Mycobacterium tuberculosis* Beijing genotype strains associated with resistance to streptomycin: a population-based study. PloS One. 2012; 7: e42323.
20. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, *et al*. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006; 6: 23.
21. de Boer AS, Kremer K, Borgdorff MW, de Haas PE, Heersma HF, van Soolingen D. Genetic heterogeneity in *Mycobacterium tuberculosis* isolates reflected in IS*6110* restriction fragment length polymorphism patterns as low-intensity bands. J Clin Microbiol. 2000; 38: 4478-84.
22. Sun YJ, Luo JT, Wong SY, Lee AS. Analysis of rpsL and rrs mutations in Beijing and non-Beijing streptomycin-resistant *Mycobacterium tuberculosis* isolates from Singapore. Clin Microbiol Infect. 2010; 16: 287-9.
23. Lai CC, Tan CK, Lin SH, Liao CH, Huang YT, Chou CH, *et al*. Clinical and genotypic characteristics of extensively drug-resistant and multidrug-resistant tuberculosis. Eur J Clin Microbiol Infect Dis. 2010; 29: 597-600.

24. Jou R, Chiang CY, Huang WL. Distribution of the Beijing family genotypes of *Mycobacterium tuberculosis* in Taiwan. J Clin Microbiol. 2005; 43: 95-100.
25. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, *et al*. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis. 2009; 13: 900-6.
26. Lawn SD, Nicol MP. Xpert(R) MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiol. 2011; 6: 1067-82.
27. Kremer K, van Soolingen D, Frothingham R, Haas WH, Hermans PW, Martin C, *et al*. Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. J Clin Microbiol. 1999; 37: 2607-18.
28. Comas I, Homolka S, Niemann S, Gagneux S. Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies. PloS One. 2009; 4: e7815.
29. Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, *et al*. High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. PLoS Biol. 2008; 6: e311.
30. Kremer K, van-der-Werf MJ, Au BK, Anh DD, Kam KM, van-Doorn HR, *et al*. Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. Emerg Infect Dis. 2009; 15: 335-9.
31. de Steenwinkel JE, Ten Kate MT, de Knegt GJ, Kremer K, Aarnoutse RE, Boeree MJ, *et al*. Drug Susceptibility of *Mycobacterium tuberculosis* Beijing Genotype and Association with MDR-TB. Emerg Infect Dis. 2012; 18: 660-3.

CHAPTER 8

Clustering of Beijing genotype *Mycobacterium tuberculosis* isolates from the Mekong delta in Vietnam on the basis of variable number of tandem repeat versus restriction fragment length polymorphism typing

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BMC Infectious Diseases 2013, 13:63

Abstract

Background
In comparison to restriction fragment length polymorphism (RFLP) typing, variable number of tandem repeat (VNTR) typing is easier to perform, faster and yields results in a simple, numerical format. Therefore, this technique has gained recognition as the new international gold standard in typing of Mycobacterium tuberculosis. However, some reports indicated that VNTR typing may be less suitable for Beijing genotype isolates. We therefore compared the performance of internationally standardized RFLP and 24 loci VNTR typing to discriminate among 100 Beijing genotype isolates from the Southern Vietnam.

Methods
Hundred Beijing genotype strains defined by spoligotyping were randomly selected and typed by RFLP and VNTR typing. The discriminatory power of VNTR and RFLP typing was compared using the Bionumerics software.

Results
Among 95 Beijing strains available for analysis, 14 clusters were identified comprising 34 strains and 61unique profiles in 24 loci VNTR typing ((Hunter Gaston Discrimination Index (HGDI = 0.994)). 13 clusters containing 31 strains and 64 unique patterns in RFLP typing (HGDI = 0.994) were found. Nine RFLP clusters were subdivided byVNTR typing and 12 VNTR clusters were split by RFLP. Five isolates (5%) revealing double alleles or no signal in two or more loci in VNTR typing could not be analyzed.

Conclusions
Overall, 24 loci VNTR typing and RFLP typing had similar high-level of discrimination among 95 Beijing strains from Southern Vietnam. However, loci VNTR 154, VNTR 2461 and VNTR 3171 had hardly added any value to the level of discrimination.

Background

The IS*6110* restriction fragment length polymorphism (RFLP) typing was previously considered the gold standard in the molecular epidemiology of tuberculosis [1]. Although this typing technique generally revealed a high level of discrimination among *Mycobacterium tuberculosis* isolates, it was considered complicated, technically demanding, and time consuming. In addition, a part of the strains contained too few copies of IS*6110* to enable a reliable typing. Variable number of tandem repeat (VNTR) typing is easier and faster to perform, and yields results in a numerical format. Therefore, this technique has become the new international typing method for *M. tuberculosis* since 2006 [2]. Several studies indicated that VNTR typing is as discriminative as RFLP typing and more suitable to type strains with few copies of IS*6110* [3,4]. However, doubt remained whether VNTR typing is as good as RFLP typing in discriminating Beijing genotype strains. As in Vietnam about 40% of the *M. tuberculosis* isolates are of this genotype, we in this study compared the performance of RFLP and internationally standardized 24 loci VNTR typing to discriminate among one hundred Beijing genotype isolates from the South of Vietnam.

Methods

Study population

In total 100 *M. tuberculosis* isolates of the Beijing genotype family were selected from a previous study on the dynamics of tuberculosis transmission in Vietnam. The study area consisted of three adjacent rural districts in Tiengiang Province, in the Mekong River Delta in Southern Vietnam. All patients were aged ≥15 years, resident in the study area and registered for treatment of smear-positive pulmonary tuberculosis (TB) between 1 January 2003 and 28 June 2007 at the participating District Tuberculosis Units, or at the provincial TB hospital and were eligible for inclusion into the study. Each eligible patient submitted two sputum samples for TB culture, drug susceptibility testing and genotyping and completed an interview form. The details of this study have been published previously [5].

Ethical approval

Ethical clearance was obtained from the ethical health committee of the Ho Chi Minh City Council (reference number 1106/UBND-VX). All included patients provided written informed consent.

***Mycobacterium tuberculosis* culture**

Sputum specimens were kept refrigerated and transported to Pham Ngoc Thach Hospital in Ho Chi Minh City within 72 hrs after collection. They were decontaminated and liquefied using 1% N-acetylcysteine/2% NaOH, inoculated on modified Ogawa medium and incubated at 37^0C. Cultures were examined for growth after 1, 2, 4, 6 and 8 weeks of incubation. Cultures with no growth after 8 weeks were considered negative. *M. tuberculosis* was identified using the niacin and the nitrate tests [6].

DNA typing

Genomic DNA was extracted from positive cultures using an earlier described method [7]. IS*6110* RFLP typing and spoligotyping were performed according to the internationally standardized methods [1,8]. The VNTR typing was executed on the basis of 15 loci and 24 loci as described by Supply *et al*.[2].

Random selection of one hundred Beijing genotype strains

Among 1,797 *M. tuberculosis* isolates that were successfully typed in RFLP and spoligotyping, 819 strains represented the Beijing genotype according to spoligo typing patterns. After the isolate numbers had been sorted in numerical order, every 8th Beijing genotype was selected until a total of 100 isolates was reached.

Data analysis

Gene Marker software, version 1.5 (Softgenetics, PA, USA) was used for analysis and automated allele calling of the VNTR patterns. The Bionumerics software, version 3.0 (Applied Maths, Sint-Martens Latem, Belgium) was used for the analysis and comparison of IS*6110* RFLP and VNTR typing patterns.
The Hunter Gaston Discrimination Index (HGDI) was used to analyse the discrimination power of VNTR and RFLP typing results [9]:

$$D = 1 - \frac{1}{n(n-1)} \sum_{j=1}^{S} nj(nj-1)$$

Where n is the total number of strains in the sample population, s is the total number of types described, and nj is the number of strains belonging to the jth type. This equation is derived as follows: the probability that a single strain sampled at random will belong to the jth group is nj/n and the probability that two strains sampled consecutively will belong to that group is $nj(nj - 1)/n(n - 1)$.

Definitions

Beijing lineage (genotype) strains were defined as strains having at least three of the nine spacers 35 to 43 and lacking spacers 1-34 based on the 43 spacer spoligo patterns [8,10]. If a strain missed all spacers 1-34 and also one or a few of the spacers 35-43, the Beijing strains was considered to represent the Atypical branch of the Beijing genotype lineage [11].

Two strains were defined as a cluster if they had identical RFLP patterns or identical VNTR profiles (Bionumerics), or if VNTR types differed by no more than a single locus [2].

Results

In the period January 2003 to June 2007, a total of 2,664 *M. tuberculosis* strains were isolated from eligible patients, of which 1,795 were successfully typed in RFLP and spoligo typing. Of these, 819 (45.6%) were of the Beijing genotype based on the spoligo patterns; the remaining 976 (54.4%) were of other genotypes. Among the 819

Beijing genotype strains, 41 (5.0%) most likely belonged to Atypical Beijing lineage, as they missed one or more spacers of the characteristic 9 spacer signature.

Of the 819 Beijing strains, 353 (43.1%) were isolated from patients in the Cailay district; 221 strains (27%) isolated from patients in the Caibe district, and the remaining 245 strains (29.9%) from patients in the Chauthanh district. Regarding the gender of patients, 592/819 (72.3%) of the strains were isolated from male patients, the remaining 227 (27.7%) from females.

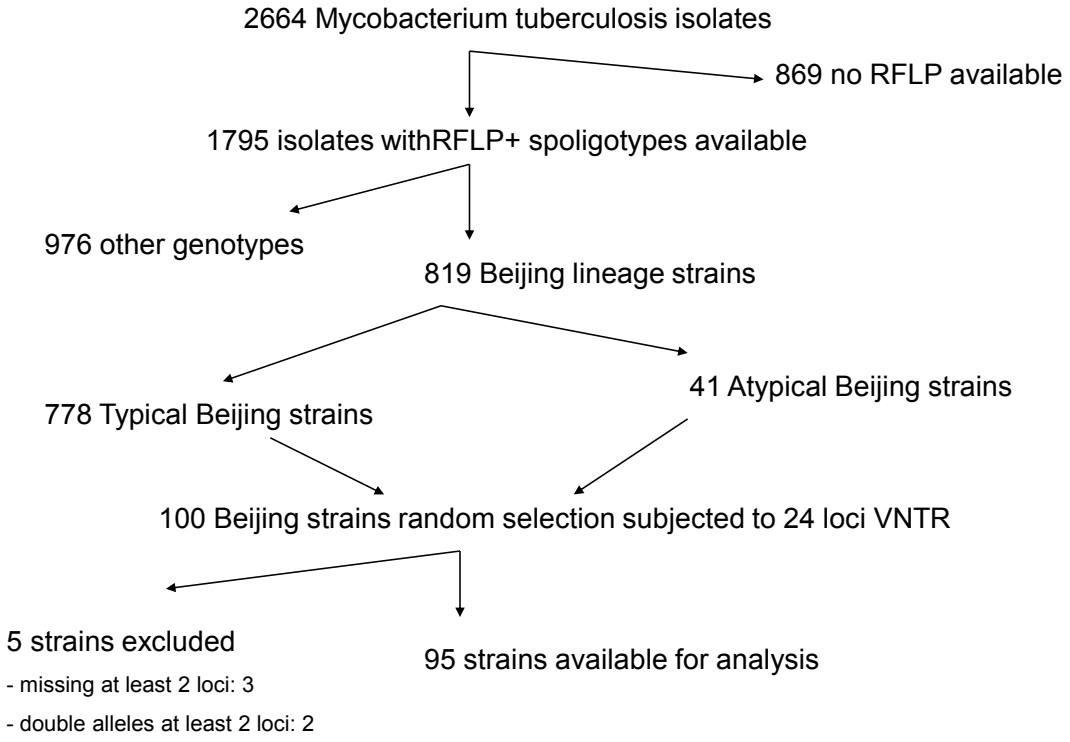

Figure 1. Study flow chart.

From these 819 Beijing genotype strains, 100 strains were randomly selected as described above, of which 5% were most likely Atypical Beijing strains because they missed one or two spacers of the characteristic nine spacer panel 35-43.

Among the 100 selected Beijing genotype strains, 38 (38%) were isolated in the Cailay district, 32 (32%) in the Caibe district and 30 (30%) in the Chauthanh district. The gender distribution was 71% males and 29% female patients. Therefore, the distribution of sex, districts, age (data not shown) in the representative Beijing strains was similar to that in the total collection of 819 Beijing strains ($P>0.05$).

Among the 100 Beijing isolates that were subjected to 24 loci VNTR typing, 95 yielded results suitable for analysis (including 88 that yielded results for all 24 loci and 7 with double alleles or that missed one locus only), the remaining 5 strains were excluded because ambiguous PCR results were obtained or double alleles were

observed in at least two loci. Among 3/5 isolates at least two loci could not be amplified and two isolates had double alleles in two or more loci (Figure 1).

Of the 24 loci analyzed, VNTR 154, VNTR 2461 and VNTR 3171 had no discrimination power and hence, the HGDI was zero. Loci VNTR 2347, VNTR 580, VNTR1644, VNTR 0577, VNTR 2531, VNTR 2401 and VNTR 802 had a HGDI of less than 0.2. The loci having a HGDI of more than 0.4 were VNTR 424, VNTR 960, VNTR 2996, VNTR 4052, VNTR 1955, VNTR 2165 and VNTR 2163b. Locus VNTR 2163b had the highest allelic diversity, with a HGDI of 0.64 (Figure 2 and Table 1).

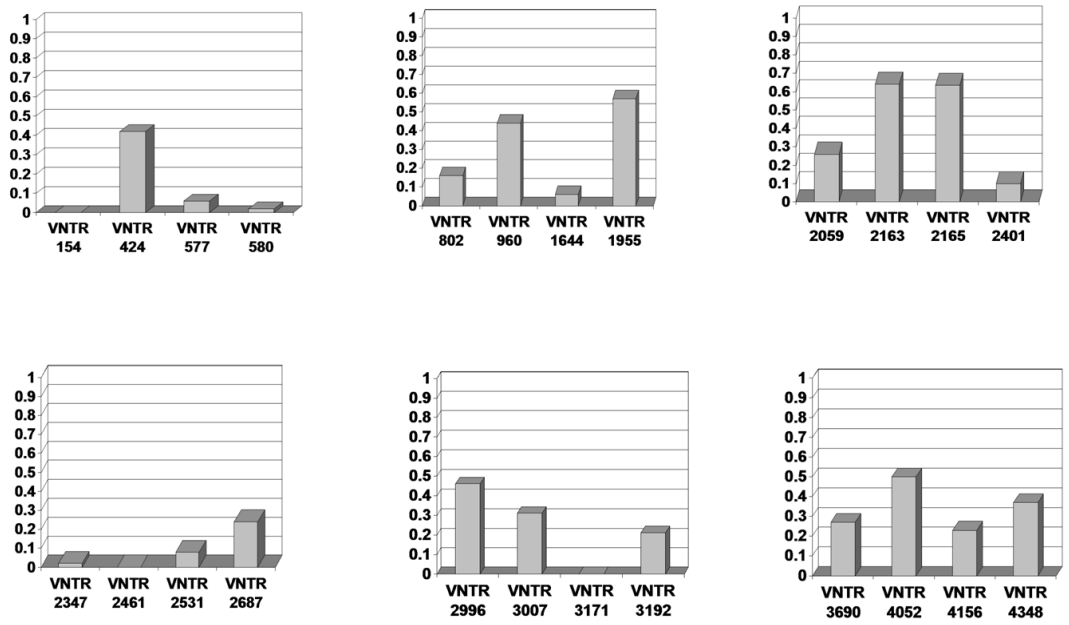

Figure 2. Hunter Gaston discrimination index of 24 VNTR loci among 95 Beijing strains. Y axis: Hunter Gaston discrimination index.

In the 24 loci VNTR typing of the remaining 95 isolates, 14 clusters of isolates were found; one cluster of four isolates, four clusters of three isolates and nine clusters of two strains. Sixty-one isolates revealed a unique VNTR pattern. The HGDI amounted to 0.994. Twelve of the VNTR clusters were subdivided in RFLP typing.

In RFLP typing, 13 clusters of isolates were found, comprising in total of 31 strains. There were ten clusters of two, two clusters of three and one cluster of five isolates. Sixty-four isolates had a unique RFLP pattern (HGDI = 0.994). Nine RFLP clusters were subdivided in VNTR typing.

Discussion

From 1993 to 2006, RFLP typing was considered the gold standard in typing of *M. tuberculosis* isolates, especially for strains harboring multiple IS*6110* copies, like the ones of the Beijing genotype family. However, this typing method is technically demanding and time consuming [4]. Furthermore, the discriminatory power of RFLP typing among strains with a low number of IS*6110* copies (<= 5 copies) is very poor

[3]. Therefore, in recent years VNTR typing has increasingly been explored in the molecular epidemiology worldwide and with the proposal on international standardization of this technique in 2006 it has in fact become the new gold standard [2]. VNTR typing introduced major advantages in typing in comparison to RFLP typing, such as its ease in use, its suitability for standardization, and that the results that are displayed in numbers can be analyzed easily and exchanged efficiently between laboratories. Moreover, the turnaround time of VNTR typing is much shorter than that of RFLP typing, because it is PCR-based and only a little amount of mycobacterial DNA is required.

Table 1. Hunter Gaston discrimination index (HGDI) values obtained for each locus in the present compared to two other studies.

VNTR locus	Present study	Kremer *et al.* [4]	Alonso *et al.* [12]
154	0.00		0.23
2461	0.00	0.00	
3171	0.00		
2347	0.02		
580	0.02	0.019	0.21
1644	0.06	0.058	0.455
577	0.06	0.165	0.63
2531	0.08		0.655
2401	0.10		0.65
802	0.16	0.196	0.73
3192	0.21		0.36
4156	0.23		0.53
2687	0.24		0.06
2059	0.26		0.16
3690	0.27		0.64
3007	0.31		0.13
4348	0.37	0.32	0.09
424	0.42		0.66
960	0.44	0.377	0.685
2996	0.46	0.2	0.46
4052	0.50	0.299	0.8
1955	0.57		0.65
2165	0.635	0.201	0.61
2163b	0.64	0.618	0.78

Many researchers have carried out comparisons between RFLP and 12 or 15 loci VNTR typing methods for discriminating *M. tuberculosis* isolates [3,4,12]. Their findings showed that the discriminative power of 12 loci VNTR was lower than that of 15 loci VNTR (with HGDI of 0.978-0.995) [12] and 15 loci VNTR has high level of

discrimination with HGDI 0.990-0.995 [4,12], but this was still lower than that of RFLP typing (0.998) [4]. However, Supply *et al*. [2] proposed to apply 24 instead of 15 loci in VNTR typing and this improved the level of discrimination significantly.

In our study, we compared the performance of 15 and 24 loci VNTR typing and RFLP typing using 95 Beijing strains and we found that the discrimination index (HGDI) of 15 loci VNTR was the lowest (0.992), followed by both RFLP typing and 24 loci VNTR typing (0.994). However, the differences observed were small. The HGDI of some loci (VNTR 154, VNTR 2461, VNTR 3171) were low in our study, which means that these loci are less useful in discriminating Beijing strains in the South of Vietnam and presumably elsewhere. The HGDI of VNTR 2461, VNTR 577, VNTR 2163b, VNTR 580, VNTR 802, VNTR 960, VNTR 1644 and VNTR 4348, were similar to that observed in a previous study in Hong Kong [4] (Table 1), whereas the HGDI of VNTR 2996, VNTR 4052, and VNTR 2165 were significantly higher than the ones in that study [4] (Table 1), for unknown reasons. It may be that because BCG vaccination has been introduced much earlier in Hong Kong than in Vietnam, the ongoing selection of particular strains of the Beijing lineage [11] may be more advanced in the former than in the latter area and the mentioned loci may have a different level of discriminative power among the circulating strains in both areas.

Our study further found the HGDI of VNTR 1955, 2163b and 2165 to be very high (>0.50) and the best differentiation, similar to two previous studies [4,12], was obtained with VNTR 2163b (Table 1 and Figure 2).

Some of the HGDI of individual loci in our study were significantly different to the ones found in the study of Alonso *et al.* [12] (Table 1), because we performed VNTR typing of exclusively Beijing strains, whereas Alonso *et al.* [12] carried out VNTR typing on a strain collection consisting of 32% LAM, 28% Haarlem and only 2% Beijing strains.

A disadvantage of VNTR typing encountered in this study was that six strains revealed double alleles in a single locus, and two strains even in two and more than two loci. It is not clear whether the latter observation was associated with a mixed infection [13]. However, the revealed genomic instability in particular loci decreases the utility of VNTR typing significantly, as this hampers a reliable interpretation. Also in RFLP typing transposition of IS*6110* sometimes interfered with a reliable interpretation, but such a genetic turn-over was observed less frequently [14]. However, we cannot exclude the possibility that these multiple alleles may reflect important phenomena in the epidemiology of TB currently unknown, and these observations, although technically demanding, may be associated with the ongoing adaptation of *M. tuberculosis* to the current TB control measures.

A major limitation of this study was that we did not have epidemiological information available to verify the transmission links indicated by both typing methods. It was therefore, not possible to ascertain the validity of epidemiological links indicated.

Conclusions

In comparison to 15 loci VNTR, RFLP typing and 24 loci VNTR typing revealed the highest level of discrimination among 95 isolates of the Beijing genotype from

Southern Vietnam. For this and other practical reasons, the last method is preferred in investigations on transmission of Beijing strains in Vietnam. The VNTR typing method is in principle also useful in screening for possible mixed infections, after which positive findings (more than two loci with double alleles) would be confirmed by other methods.

Acknowledgements

This research was funded by the KNCV Tuberculosis Foundation, the Medical Committee Netherlands-Vietnam, The Netherlands.

We thank all TB patients participating in this study and the staff of the National Tuberculosis Program of Tien Giang province for recruiting the patients as well as the staff of the National Tuberculosis Program in PNT hospital for delivering, supervising and checking the data. Dai Viet Hoa, Phan Thi Hoang Anh and other staff in Pham Ngoc Thach laboratory are deeply thanked for performing culture, DST and GenoType® MTBDR*plus* testing. We are grateful to Anne-Marie van den Brandt, Mirjam Dessens Mimount Enaimi and Arnout Mulder, Jessica de Beer and other staff of the Tuberculosis Reference Laboratory at the National Institute for Public health and the Environment (The Netherlands) for providing us the knowledge and practice on the molecular techniques used in this study.

References

1. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, *et al*: Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol 1993, 31:406-9.
2. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, *et al*: Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. J Clin Microbiol 2006, 44:4498-510.
3. Smittipat N, Billamas P, Palittapongarnpim M, Thong-On A, Temu MM, Thanakijcharoen P, *et al*: Polymorphism of variable-number tandem repeats at multiple loci in *Mycobacterium tuberculosis*. J Clin Microbiol 2005, 43:5034-43.
4. Kremer K, Au BK, Yip PC, Skuce R, Supply P, Kam KM, *et al*: Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS*6110* restriction fragment length polymorphism typing and spoligotyping. J Clin Microbiol 2005, 43:314-20.
5. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, *et al*: The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis 2009, 13:900-6.
6. International Union Against Tuberculosis and Lung Disease: Technical guide-sputum examination for tuberculosis direct microscopy in low-income countries. 5th ed. Paris: The Union, 2000.
7. van Soolingen D, de Haas PEW, Kremer K: Restriction fragment length polymorphism typing of mycobacteria. In Mycobacterium tuberculosis protocols. Edited by T. Parish, Stoker NG. Totowa, New Jersey, USA: Humana Press Inc.; 2001: 165-203.
8. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, Bunschoten A, Molhuizen H, Shaw R, Goyal M *et al*: Simultaneous detection and strain

differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol 1997, 35:907-14.

9. Hunter PR, Gaston MA: Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol 1988, 26:2465-66.
10. Kremer K, Glynn JR, Lillebaek T, Niemann S, Kurepina NE, Kreiswirth BN, *et al*: Definition of the Beijing/W lineage of *Mycobacterium tuberculosis* on the basis of genetic markers. J Clin Microbiol 2004, 42:4040-9.
11. Kremer K, van-der-Werf MJ, Au BK, Anh DD, Kam KM, van-Doorn HR, *et al*: Vaccine-induced immunity circumvented by typical *Mycobacterium tuberculosis* Beijing strains. Emerg Infect Dis 2009, 15:335-9.
12 Alonso-Rodriguez N, Martinez-Lirola M, Herranz M, Sanchez-Benitez M, Barroso P, Bouza E, *et al*: Evaluation of the new advanced 15-loci MIRU-VNTR genotyping tool in *Mycobacterium tuberculosis* molecular epidemiology studies. BMC Microbiol 2008, 8:34.
13. Shamputa IC, Jugheli L, Sadradze N, Willery E, Portaels F, Supply P, *et al*: Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respir Res 2006, 7:99.
14. de Boer AS, Borgdorff MW, de Haas PE, Nagelkerke NJ, van Embden JD, van Soolingen D: Analysis of rate of change of IS*6110* RFLP patterns of *Mycobacterium tuberculosis* based on serial patient isolates. J Infect Dis 1999, 180:1238-44.

CHAPTER 9

GENERAL DISCUSSION

New molecular methods are useful for early diagnosis of MDR-TB in Vietnam

We re-cultured 111 MTB MDR-TB, rifampin-resistant- or pan-susceptible *M. tuberculosis* isolates according to conventional DST and tested these with the GenoType® MTBDR*plus* test. We found a high specificity and positive predictive value of the GenoType® MTBDR*plus* test for MDR-TB (Chapter 2); our results were similar to some previous studies in other geographic areas [1, 2].

Early diagnosis of multidrug resistant tuberculosis (MDR-TB) plays an important role in interruption of MDR-TB transmission and in improving treatment outcome, whereas the conventional drug susceptibility testing requires 2 to 4 months for results.

The Genotype® MTBDR*plus* test is one of the rapid and commercially available drug susceptibility tests that have been introduced in various countries for routine diagnosis of MDR-TB [1, 3-5]. This test has a high sensitivity and a very high specificity, in test situations and in routine use, as we showed in this thesis, and which was also found by others [1, 2]. Given the high sensitivity and specificity as well as the associated high positive and negative predictive values, the test has greatly reduced the diagnostic delay for MDR-TB patients.

In September 2009, the National TB Program (NTP) in Vietnam approved routine use of this test in two TB reference laboratories for the rapid diagnosis of MDR-TB in patients suspected of MDR-TB. Also, the NTP provided free of charge MDR-TB treatment to MDR-TB patients diagnosed by the GenoType® MTBDR*plus* test. While we tested an older version of the test that was designed to test smear-positive sputum samples, recently a new version of the test that can also be used for smear negative TB patients was released. However, appropriate use of the GenoType® MTBDR*plus* test requires skilled technicians, three separate rooms (for preparation of the PCR mix, DNA extraction, and amplification); and a 24-hour turnaround time. For these reasons, this test is not suitable for use in district laboratories.

In late 2009, the Cepheid company released the GeneXpert MTB/RIF assay, a nucleic acid-amplification test (NAAT) for *M. tuberculosis* detection and MDR-TB screening with high sensitivity and specificity [6]. Contrary to the GenoType® MTBDR*plus* test, the GeneXpert MTB/RIF can be performed by low skilled technicians in a peripheral laboratory and it can be applied to smear negative samples within a 2-hour turnaround time [6]. With such advantages, the GeneXpert MTB/RIF is becoming a common test around the world for the detection of MDR-TB. However, compared to the GenoType® MTBDR*plus* test, the disadvantages are that the test is not able to detect rifampicin resistance in mixed samples of resistant and sensitive strains, in which the concentration of resistant strain is less than 65% of the bacteria in the whole isolate [7], and that it gives a rifampicin resistance result, if the band WT8 is absent and the *rpoB* MUT3 probe does not develop, a such combination may be a L533P mutation that shows sensitivity to rifampicin in conventional tests [2].

Recently, the MTBDR*sl* test has been introduced to diagnose XDR-TB, with high sensitivity and specificity for detection of fluoroquinolone and aminoglycoside resistance [8, 9]. Since 2012, this test has been used as a routine test to exclude XDR-TB among MDR-TB patients in the STREAM study in Vietnam.

Thanks to these tests at least a part of the MDR-TB patients will be treated with appropriate drugs, therefore the transmission of MDR-TB and XDR-TB in the community will be reduced.

The variable number of tandem repeat (VNTR) typing is a faster and easier tool than restriction fragment length polymorphism (RFLP) typing for tracking of TB transmission and for detecting mixed infections

Hundred Beijing genotype strains, as defined by spoligotyping, were randomly selected and typed by RFLP and VNTR typing. The discriminatory power of VNTR and RFLP typing was compared using the Bionumerics software. Overall, 24 loci VNTR typing and RFLP typing had similar high-levels of discrimination among 95 Beijing strains from Southern Vietnam (Chapter 8).

Previous studies indicated that VNTR typing is as discriminative as RFLP typing and more suitable to type strains with few copies of IS*6110* [10, 11]. The 24 loci VNTR is also reliable in discriminating MTB strains having high number of IS*6110* copies in their genome, such as Beijing genotype (Chapter 8, [11]). Therefore, 24 loci VNTR typing is an appropriate method for epidemiological investigation of TB transmission in Vietnam, where East African Indian genotype (few IS*6110* copies) and Beijing genotype are two most predominantly circulating lineages [12]. However, the revealed genomic instability in particular loci decreases the utility of VNTR typing significantly, as this hampers a reliable interpretation. It remains to be determined how many different loci or how many loci having double alleles should be considered indications of two different strains, because different loci or double alleles in few loci could also represent the regular evolutionary development of the bacterium.

Mixed infections are prevalent in Vietnam and patients with multiple infections could have an increased immunological tolerance to M. tuberculosis infections.

In Chapter 3, 1,248 *M. tuberculosis* isolates from the same number of patients were subjected to RFLP-, spoligo- and VNTR typing. A patient was defined as having a mixed infection when his/her *M. tuberculosis* isolate exhibited RFLP and spoligotype patterns of two different *M. tuberculosis* lineages.

Mixed infections were confirmed in 39 (3.1%) patients; all were cured by standard treatment. Mixed infections occurred more frequently in new than in re-treatment patients, were significantly associated with minor X-ray abnormalities and there was a near-to-significant trend for lower sputum smear grades, both suggesting association with less extensive pathology. Simultaneous infection with two strains of *Mycobacterium tuberculosis* was previously detected by applying phage typing or genotyping techniques such as the IS*6110* RFLP-, spoligo- and VNTR typing [13-18] (Chapter 3 and Chapter 8). The Genotype® MDRTB*plus* assay, can in principle also diagnose mixed infections of drug sensitive and drug resistant strains (Chapter 2).

Based on RFLP and spoligotyping, we revealed that the rate of mixed infections of strains of two distinct lineages in a rural area in Vietnam with a population density of only 837/km^2 and an observed TB incidence of new smear positive cases of 100/100,000 in 2005 (National Tuberculosis Program Vietnam, unpublished data) and a

very low (0.5%) prevalence of HIV [19], 3.1% was lower than the rates reported in similar studies in South Africa, where the prevalence of TB and HIV are very high [20,14]. Our results show that mixed infections can also occur in an area with an incidence of TB of about 100/100000 population per year, with a low HIV co-infection rate, and that mixed infections are not related to TB symptoms or drug resistance.

With 100% of the cases being cured, we do not have an indication that mixed infections lead to higher failure rates. In fact, in our population, they were associated with less extensive chest X-ray abnormalities than single infections. Moreover, mixed infections were associated with a lower degree of smear positivity; one hypothesis could be that patients with multiple infections have an increased immunological tolerance to *M. tuberculosis* infections. This finding, however, requires further study (Chapter 3).

Besides Beijing and East African Indian strains, also strains without* IS*6110* *are prevalent in Vietnam; therefore* IS*6110*-*based PCR is unreliable in TB diagnosis

Consecutively diagnosed adult TB patients in rural Southern Vietnam submitted two sputum samples for culture, IS*6110* RFLP-, spoligo- and 15 loci VNTR typing. PCR was performed to confirm the absence of the IS*6110* elements in strains lacking IS*6110* hybridization in RFLP. Among 2,664 TB patient isolates examined, 109 (4.1%) had no IS*6110* element. Compared to other strains, these no-copy strains were less often resistant to anti-tuberculosis drugs, especially to streptomycin (adjusted odds ratio 0.2, 95% CI: 0.1-0.5), and showed significant variation in geographic site of isolation. No associations with TB history or demographic factors were found. Hence, strains without the IS*6110* target pose a problem in Vietnam regarding false-negative molecular TB diagnosis in PCR (Chapter 7). Based on spoligotyping, Brudey *et al.* found many different genotypes of MTB occurring worldwide [21], in which Beijing genotype is the best known genotype, because it was not only encountered in many countries with high prevalence especially in East and South East Asia [22-25], but also associated with drug resistance and MDR [22, 26-30].

From 1,084 MTB isolates collected in a nationwide drug resistance survey done in 2005 in Vietnam, Hung *et al.* found that 35% was of the Beijing genotype and 44.4% of the East African Indian (EAI) lineage. The Beijing genotype was more frequent in the South than in the North of Vietnam (39% vs. 30%, respectively) [12]. Besides the Beijing and EAI genotypes, the no-copy was considered more frequent in Asia, especially South East Asia [31]. In our study, 4.1% of the TB patients was infected with no-copy strains in a rural area of Vietnam (Chapter 7); this rate is significantly lower than the 11% reported previously in India [32]. This difference is probably due to the differences in geographical distribution of genotypes of *M. tuberculosis*, but also in identification and verification of no-copy strains. We used RFLP and spoligotyping to screen for IS*6110*-devoid strains and used PCR to confirm IS*6110* PCR for verification. It is conceivable that this approach has a higher specificity for detecting no-copy strains, resulting in fewer misclassifications of strains with IS*6110* bands as no-copy strains, than when only RFLP typing is applied (Chapter 7).

The characteristics of no-copy strains were completely opposite to those of Beijing strains. The huge differences between the no-copy and the Beijing genotype strains in correlation with factors like resistance to tuberculosis drugs may be due to the fact that the latter belong to the modern lineage, which have undergone more mutations, whereas the former seem to belong to an earlier ancestor of the *M. tuberculosis* complex [33, 34]. Furthermore, the Beijing genotype strains themselves may have a much higher intrinsic resistance to treatment by rifampicin, as well as a higher mutation frequency regarding the generation of rifampicin resistant mutants [35].

In 2002, Lok *et al.* showed that the prevalence of no-copy strains among TB patients with genotyping results was 0.18% in European countries [31], compared to Asian countries, where about 11% of all genotyped isolates concerns IS*6110*-devoid strains [32], while this was 4.1% in a rural area of Vietnam (Chapter 7). To our knowledge, so far, nowhere in the world, an outbreak has been reported caused by a no-copy genotype strain. This genotype mostly occurs among elderly patients aged 65 years and above, who are believed to develop TB disease mainly due to endogenous reactivation of remote infections rather than by acquisition of a recent infection [8]. This most likely indicates that no-copy strains are in terms of evolution less fit to deal with the current measures against TB, and will disappear in the coming decades. Alternatively, they may lose IS*6110* DNA during latency in the patients.

Since in our no-copy strain study, 4% of all isolates found in a population-based sample in rural South Vietnam represented a no-copy strain, this urges the IS*6110* PCR to be replaced by another PCR method not using IS*6110* as a target, for instance a specific *rpoB* gene signature present in all *M. tuberculosis* complex strains, such as in the GeneXpert® MTB/RIF (Chapter 7).

In Vietnam, patients infected with MTB resistant to isoniazid, have an increased risk of poor treatment outcomes

By use of the Genotype® MTBDR*plus* test, we found that in the rural South Vietnam *katG* codon 315 mutations were most frequent (Chapter 2, Chapter 6). Among 251 isoniazid resistant strains, 75.3 % revealed *katG* codon 315 mutations (Chapter 6); this rate was similar to some previous reports in South Africa (64.1%, 72%), respectively [1, 36]. Both *katG* codon 315 and *inhA* promoter region mutations are strongly associated with relapse, and *katG* codon 315 mutations were also associated with unfavorable treatment outcome (treatment failure and death). In addition, *katG* codon 315 mutations were strongly related to rifampicin, streptomycin and ethambutol resistance, which was similar to the findings in two previous studies [37, 38]. These findings raise the hypothesis that mutations in codon 315 of *katG* gene may trigger the acquisition of other mutations conferring resistance to other drugs such as rifampicin, streptomycin, and ethambutol. Alternatively, the reduction in fitness in 315 mutants may be lower than for other mutants, yielding more persistent infections and a higher chance of developing additional resistance.

Against strains with such INH resistance mutations, the regimen 2SHRZ/6EH is far less effective, which may further elucidate why these mutations are associated with unfavorable treatment outcome in our study (Chapter 6) and in Tolani's study [37]. As

mentioned in this thesis, the 2SHRZ/6EH regimen used in Vietnam may be one of the reasons for the high rate of relapse in this country (Chapter 4 and Chapter 6). The 2010 WHO TB treatment guidelines advised to replace 8-month regimens by a 6-month regimen (2HRZE/4RH) with rifampicin throughout. In populations with known or suspected high levels of isoniazid resistance, new TB patients may receive HRE as therapy in the continuation phase as an acceptable alternative to HR [39]. Vietnam is a country with high levels of isoniazid resistance in new TB patients [40], making implementation of the 2HRZE/4RHE regimen for treatment of new TB patients necessary. In early July 2013, the Vietnamese National TB program decided to apply the 6-month regimen (2HRZE/4RHE) to new TB patients in Ho Chi Minh City, where a high prevalence of Beijing genotype strains exists and this regimen may be used in the whole country in the near future (National TB Program, unpublished data).

In previous studies, Buu *et al.* conceded that streptomycin resistance, a prerequisite for the outbreak of MDR-TB Beijing strains in Vietnam, played an important role in the association between Beijing genotype and MDR-TB, and increased the probability of transmission of the Beijing genotype strains [41]. In Chapter 6, likewise, we disclosed that MTB-TB strains having *katG* codon 315 mutations are more frequent among Beijing genotype strains that are also resistant to streptomycin. Taken together, these findings suggest that streptomycin may be ineffective for treatment of TB in patients infected with Being genotype or isoniazid resistant MTB strains. Thus, the 2HRZE/4HRE regimen should be used in settings where Beijing genotype strains are circulating at high prevalence like in Vietnam.

The Beijing genotype is one of the risk factors causing relapse in Vietnam; therefore, the 2SHRZ/6EH regimen needs to be replaced

Beijing strains are one of the two common MTB genotypes in Vietnam, making up 35% of all genotypes found in rural South Vietnam [29]; the Beijing genotype is more frequent in the South than in the North of Vietnam (39% vs. 30%, respectively) [12]. These strains are associated with young people, streptomycin and isoniazid resistance as well as with MDR-TB, relapse and treatment failure [22, 29, 42]. The rate of relapse in patients infected with Beijing strains was 5.5 times higher than in patients infected with other MTB strains and this association was independent of TB treatment history and pretreatment drug resistance pattern. Furthermore, in our relapse study (Chapter 4), relapse was significantly associated with resistance to isoniazid, suggesting that the use of a combination of isoniazid and ethambutol only in the continuation phase of treatment seems to be ineffective to kill Beijing strains, as also shown by Jindani *et al.* [43], (Chapter 4, Chapter 6).

Increased relapse rates may in fact reflect increased failure rate with very low bacterial loads, resulting in negative cultures at the end of treatment but increasing numbers of bacilli once drug treatment is stopped. This may explain why we find shorter intervals between cure and relapse in cases caused by Beijing strains. However, in our failure study (Chapter 5), we found no association between genotype and treatment failure, and no other study clearly did so, except for a study in Indonesia [44]. In our relapse study, we found that the average time from cure to relapse was shorter in

patients infected with Beijing genotype strains than in patients infected with other strains: 10.5 versus 15.8 months (Chapter 4). Indeed, based on results of a study from The Gambia, De Jong and colleagues suggested that Beijing strains have higher rates of progression from latent infection to disease [45].

For Vietnam, an increased relapse rate of Beijing strains seems to be an extra reason to move from the 8-month to the recommended 6-month regimen [39]. In addition, if the increased relapse rate reflects increased rates of treatment failure, this may be a reason to strengthen the continuation phase of the treatment regimen for new TB patients in Vietnam, e.g. by adding ethambutol to the 4-month continuation phase (2HRZE/4HRE) (Chapter 4 and Chapter 6).

CONCLUSIONS AND RECOMMENDATIONS FOR IMPROVING TUBERCULOSIS CONTROL IN VIETNAM

Molecular epidemiology plays an important role in identifying associations between TB genotypes and treatment outcome or/and clinical characteristics. It greatly improved our understanding of the transmission dynamics of *M. tuberculosis* within different populations. Furthermore, molecular techniques aided in discovering mutations conferring resistance to TB drugs for early diagnosis MDR-TB and XDR-TB. In 2009, we applied the GenoType® MTBDR*plus* test in routine applications to diagnose suspected MDR-TB patients and to treat such patients with an appropriate regimen. This is an important turning point that effectively supports the National TB program in controlling and treating MDR-TB patients.

Based on our findings in this thesis and in previous studies, we conclude that the Beijing genotype and East African Indian are two most common genotypes circulating in Vietnam. Among them, Beijing genotype plays an important role in the problems in control of tuberculosis in Vietnam.

Streptomycin resistance is a prerequisite factor that favors Beijing genotype strains to succeed in spreading, developing drug resistance in Vietnam.

In Vietnam, nearly 90% of isoniazid resistant *M. tuberculosis* is caused by *katG* codon 315 mutations and mutations in the *inhA* promoter region, in which the former mutations are predominant. TB patients infected with *M. tuberculosis* having one or two of these mutations may develop relapse more easily. Moreover, the *katG* codon 315 mutations are linked to death and treatment failure; hence, those patients need to be strictly followed up at least 2 years after treatment.

Streptomycin is highly important in the development of MDR-TB. Therefore, streptomycin should be taken out of the eight-month regimen 2SHRZ/6EH being in use in Vietnam as a standard category 1 treatment regimen for new TB patients; the 6-month regimen 2HRZE/4HR should be applied following the WHO's guidelines [39] and could be strengthened with ethambutol in the continuation phase for settings that have high prevalence of Beijing genotype strains and isoniazid resistance, like in Vietnam.

In addition, the GeneXpert and the Genotype®MTBDR*plus* test are useful tools to control MDR-TB. These tests are now used routinely for screening of suspected MDR-TB patients in some provinces in Vietnam. To control MDR-TB more

effectively, the GeneXpert MTB/RIF or Genotype®MTBDR*plus* test should be available for all TB patients. If this becomes reality we may succeed in the battle against (resistant) tuberculosis in the near future.

References

1. Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME. Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. Am J Resp Crit Care Med. 2008;177:787-92.
2. Hillemann D, Rusch-Gerdes S, Richter E. Evaluation of the GenoType MTBDR*plus* assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. J Clin Microbiol. 2007;45:2635-40.
3. Hillemann D, Weizenegger M, Kubica T, Richter E, Niemann S. Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol. 2005;43:3699-703.
4. Hillemann D, Kubica T, Agzamova R, Venera B, Rusch-Gerdes S, Niemann S. Rifampicin and isoniazid resistance mutations in *Mycobacterium tuberculosis* strains isolated from patients in Kazakhstan. Int J Tuberc Lung Dis. 2005;9:1161-7.
5. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Performance of the genotype MTBDR line probe assay for detection of resistance to rifampin and isoniazid in strains of *Mycobacterium tuberculosis* with low- and high-level resistance. J Clin Microbiol. 2006;44:3659-64.
6. Van Rie A, Page-Shipp L, Scott L, Sanne I, Stevens W. Xpert((R)) MTB/RIF for point-of-care diagnosis of TB in high-HIV burden, resource-limited countries: hype or hope? Expert Rev Mol Diagn. 2010;10:937-46.
7. Boehme C, for the Foundation for innovative new diagnostics. Xpert MTB/RIF: Development, analytical performance, and evidence from field evaluation studies. Presented at the WHO workshop for early implementers, 7-8 April, 2011.
8. Borgdorff MW, Nagelkerke NJ, de Haas PE, van Soolingen D. Transmission of *Mycobacterium tuberculosis* depending on the age and sex of source cases. American journal of epidemiology. 2001;154:934-43.
9. World Health Organization. *M*ultidrug and extensively drug-resistant TB (M/XDR-TB). 2010 GlobalReport On Surveillance and Response. Geneva: World Health Organization, 2010.
10. Smittipat N, Billamas P, Palittapongarnpim M, Thong-On A, Temu MM, Thanakijcharoen P, *et al*. Polymorphism of variable-number tandem repeats at multiple loci in *Mycobacterium tuberculosis*. J Clin Microbiol. 2005;43:5034-43.
11. Kremer K, Au BK, Yip PC, Skuce R, Supply P, Kam KM, *et al*. Use of variable-number tandem-repeat typing to differentiate *Mycobacterium tuberculosis* Beijing family isolates from Hong Kong and comparison with IS*6110* restriction fragment length polymorphism typing and spoligotyping. J Clin Microbiol. 2005;43:314-20.
12. N.V. Hung, DNS, N.T.N. Lan, E.W. Tiemersma, F.G.J. Cobelens, D. van Soolingen. Beijing genotype distribution and association with drug resistance of *Mycobacterium tuberculosis* in Vietnam. Hanoi: National hospital of Tuberculosis and Respiratory Diseases, Hanoi, Vietnam, 2011.
13. Bates JH, Stead WW, Rado TA. Phage type of tubercle bacilli isolated from patients with two or more sites of organ involvement. Am Rev Respir Dis. 1976;114:353-8.

14. Braden CR, Morlock GP, Woodley CL, Johnson KR, Colombel AC, Cave MD, *et al*. Simultaneous infection with multiple strains of *Mycobacterium tuberculosis*. Clin Infect Dis. 2001;33:e42-7.
15. Richardson M, Carroll NM, Engelke E, Van Der Spuy GD, Salker F, Munch Z, *et al*. Multiple *Mycobacterium tuberculosis* strains in early cultures from patients in a high-incidence community setting. J Clin Microbiol. 2002;40:2750-4.
16. Shamputa IC, Jugheli L, Sadradze N, Willery E, Portaels F, Supply P, *et al*. Mixed infection and clonal representativeness of a single sputum sample in tuberculosis patients from a penitentiary hospital in Georgia. Respir Res. 2006;7:99.
17. Small PM, Shafer RW, Hopewell PC, Singh SP, Murphy MJ, Desmond E, *et al*. Exogenous reinfection with multidrug-resistant *Mycobacterium tuberculosis* in patients with advanced HIV infection. N Engl J Med. 1993;328:1137-44.
18. Yeh RW, Hopewell PC, Daley CL. Simultaneous infection with two strains of *Mycobacterium tuberculosis* identified by restriction fragment length polymorphism analysis. Int J Tuberc Lung Dis. 1999;3:537-9.
19. Thuy TT, Shah NS, Anh MH, Nghia do T, Thom D, Linh T, *et al*. HIV-associated TB in An Giang Province, Vietnam, 2001-2004: epidemiology and TB treatment outcomes. PLoS One. 2007;2:e507.
20. Cohen T, Wilson D, Wallengren K, Samuel EY, Murray M. Mixed strain *M. tuberculosis* infections among patients dying in hospital in KwaZulu-Natal, South Africa. J Clin Microbiol. 2010; 49:385-8.
21. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, *et al*. *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006;6:23.
22. Anh DD, Borgdorff MW, Van LN, Lan NT, van Gorkom T, Kremer K, *et al*. *Mycobacterium tuberculosis* Beijing genotype emerging in Vietnam. Emerg Infect Dis. 2000;6:302-5.
23. Hanekom M, Gey van Pittius NC, McEvoy C, Victor TC, Van Helden PD, Warren RM. *Mycobacterium tuberculosis* Beijing genotype: a template for success. Tuberculosis (Edinb). 2011;91:510-23.
24. Glynn JR, Kremer K, BorgdorffMW, Rodriguez MP, Soolingen DV. Beijing/W genotype *Mycobacterium tuberculosis* and drug resistance. Emerg Infect Dis. 2006;12:736-43.
25. Filliol I, Driscoll JR, Van Soolingen D, Kreiswirth BN, Kremer K, Valetudie G, *et al*. Global distribution of *Mycobacterium tuberculosis* spoligotypes. Emerg Infect Dis. 2002;8:1347-9.
26. Bifani PJ, Plikaytis BB, Kapur V, Stockbauer K, Pan X, Lutfey ML, *et al*. Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. JAMA. 1996;275:452-7.
27. Toungoussova OS, Sandven P, Mariandyshev AO, Nizovtseva NI, Bjune G, Caugant DA. Spread of drug-resistant *Mycobacterium tuberculosis* strains of the Beijing genotype in the Archangel Oblast, Russia. J Clin Microbiol. 2002;40:1930-7.
28. Drobniewski F, Balabanova Y, Nikolayevsky V, Ruddy M, Kuznetzov S, Zakharova S, *et al*. Drug-resistant tuberculosis, clinical virulence, and the dominance of the Beijing strain family in Russia. JAMA. 2005;293:2726-31.
29. Buu TN, Huyen MN, Lan NT, Quy HT, Hen NV, Zignol M, *et al*. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis. 2009;13:900-6.

30. Johnson R, Warren RM, van der Spuy GD, Gey van Pittius NC, Theron D, Streicher EM, *et al*. Drug-resistant tuberculosis epidemic in the Western Cape driven by a virulent Beijing genotype strain. Int J Tuberc Lung Dis. 2010;14:119-21.
31. Lok KH, Benjamin WH, Jr, Kimerling ME, Pruitt V, Lathan M, Razeq J, *et al*. Molecular differentiation of *Mycobacterium tuberculosis* strains without IS*6110* insertions. Emerg Infect Dis. 2002;8:1310-3.
32. Chauhan DS, Sharma VD, Parashar D, Chauhan A, Singh D, Singh HB, *et al*. Molecular typing of *Mycobacterium tuberculosis* isolates from different parts of India based on IS*6110* element polymorphism using RFLP analysis. Indian J Med Res. 2007;125:577-81.
33. Schurch AC, Kremer K, Hendriks AC, Freyee B, McEvoy CR, van Crevel R, *et al*. SNP/RD typing of *Mycobacterium tuberculosis* Beijing strains reveals local and worldwide disseminated clonal complexes. PloS One. 2011;6:e28365.
34. Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, *et al*. High functional diversity in *Mycobacterium tuberculosis* driven by genetic drift and human demography. PLoS Biol. 2008;6:e311.
35. de Steenwinkel JE, Ten Kate MT, de Knegt GJ, Kremer K, Aarnoutse RE, Boeree MJ, *et al*. Drug Susceptibility of *Mycobacterium tuberculosis* Beijing Genotype and Association with MDR-TB. Emerg Infect Dis. 2012;18:660-3.
36. World Health Oranization, Guidelines for the surveillance of drug resistance in tuberculosis. 2nd ed. WHO/CDS/2003.320. Geneva: World Health Organization, 2003.
37. Tolani MP, D'Souza D T, Mistry NF. Drug resistance mutations and heteroresistance detected using the GenoType MTBDR*plus* assay and their implication for treatment outcomes in patients from Mumbai, India. BMC infectious diseases. 2012;12:9.
38. Hazbon MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, *et al*. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2006;50:2640-9.
39. World Health Organization. Treatment of tuberculosis: guidelines for national programmes. 4th ed. WHO/HTM/TB/2009.420. Geneva: World Health Organization, 2010.
40. Huong NT, Lan NT, Cobelens FG, Duong BD, Co NV, Bosman MC, *et al*. Antituberculosis drug resistance in the south of Vietnam: prevalence and trends. J Infect Dis. 2006;194:1226-32.
41. Buu TN, van Soolingen D, Huyen MN, Lan NT, Quy HT, Tiemersma EW, *et al*. Increased transmission of *Mycobacterium tuberculosis* Beijing genotype strains associated with resistance to streptomycin: a population-based study. PLoS One.7:e42323.
42. Lan NT, Lien HT, Tung le B, Borgdorff MW, Kremer K, van Soolingen D. *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. Emerg Infect Dis. 2003;9:1633-5.
43. Jindani A, Nunn AJ, Enarson DA. Two 8-month regimens of chemotherapy for treatment of newly diagnosed pulmonary tuberculosis: international multicentre randomised trial. Lancet. 2004;364:1244-51.
44. Parwati I, Alisjahbana B, Apriani L, Soetikno RD, Ottenhoff TH, van der Zanden AG, *et al*. *Mycobacterium tuberculosis* Beijing genotype is an independent risk factor for tuberculosis treatment failure in Indonesia. J Infect Dis. 2010;201:553-7.

45. de Jong BC, Hill PC, Aiken A, Awine T, Antonio M, Adetifa IM, *et al*. Progression to active tuberculosis, but not transmission, varies by *Mycobacterium tuberculosis* lineage in The Gambia. J Infect Dis. 2008;198:1037-43.

SUMMARY

This thesis provides insight in the distribution of *Mycobacterium tuberculosis* genotypes in the rural South of Vietnam, and their relationship with anti-tuberculosis drug resistance, clinical characteristics and treatment outcome. Thanks to a combination of molecular biological and epidemiological methods, this thesis demonstrates that the GenoType® MTBDR*plus* test is a reliable tool to diagnose multidrug resistant tuberculosis (MDR-TB). Furthermore, the Beijing genotype of *M. tuberculosis* and isoniazid resistance are two predictors of relapse for TB patients. These effects may be specific for the Vietnamese situation in which patients are treated with an 8-month regimen (2SRHZ/6HE), and one could therefore speculate that this regimen may be one of the reasons for the high rate of relapse in Vietnam and should be replaced with the 6-month regimen 2HRZE/4RHE according to the WHO recommendation. Moreover, this thesis shows that mixed infections are not only present in regions with a high TB prevalence, a high density of human population and a high rate of HIV, but also in regions with a medium TB prevalence and a low rate of HIV. In addition, strains without the target IS*6110* pose a problem in Vietnam regarding false-negative molecular diagnosis in PCR IS*6110.*

Chapter 1 provides a general introduction on the burden and epidemiology of tuberculosis worldwide and in Vietnam. Also, the population structure of *M. tuberculosis* in this country is introduced, and molecular techniques for genotyping and diagnosis are summarized. Moreover, the aims of this thesis are described in this chapter.

The findings in Chapter 2, taking advantage of the nationwide TB drug resistance survey (DRS) in Vietnam conducted in 2004-2005, indicate that the GenoType® MTBDR*plus* assay has a high sensitivity, specificity, and an acceptable positive and negative predictive value for the routine diagnosis of multidrug resistant tuberculosis (MDR-TB), with a test sensitivity and specificity of 89% and 100%, respectively. Moreover, the GenoType® MTBDR*plus* assay disclosed several mixed infections of resistant and sensitive strains of *M. tuberculosis* and of non-tuberculous mycobacteria (NTM) and *M. tuberculosis*.

In Chapter 3, based on spoligotyping and restriction fragment length polymorphism (RFLP) typing, *Mycobacterium tuberculosis* strains isolated from a population-based study, we found that at least 3.1% of the TB patients in rural South of Vietnam, where TB prevalence was around 100/100.000 population in 2005, were suffering from double *M. tuberculosis* infections. Such double infections were often a mixture of a Beijing and an East African Indian strain; the two most common genotypes in Vietnam. In this study we also found that mixed infections were not associated with clinical symptoms and drug resistance. However, they were significantly less likely to occur in patients with extensive chest X-ray abnormalities and high sputum smear grades, suggesting that patients with multiple infections have an increased immunological tolerance to *M. tuberculosis* infections.

In Chapter 4 and Chapter 5, we assessed the relationship between the Beijing genotype and relapse or treatment failure, based on a prospective cohort study. In Chapter 4, we found that the relative risk of relapse after curative treatment was larger for Beijing versus non-Beijing strains (OR 5.5; 95% CI, 2.1-14.6). This rate was more

pronounced than that earlier observed in the multi-country trial of Bumman *et al.* in which patients were also monitored for recurrent TB. The higher rate of recurrent TB in cases caused by Beijing strains may be associated with the use of the 8-month 2SRHZ/6HE regimen in new patients. Because ethambutol is a bacteriostatic drug, it can prevent the growth of bacilli, but cannot kill them. So once drug treatment is stopped, the remaining bacilli may start replicating again causing symptoms and positive cultures within months. However, the Beijing genotype does not directly affect the rate of treatment failure, as mentioned in Chapter 5.

In a prospective, population-based study described in Chapter 6, we used the GenoType® MTBDR*plus* assay to detect mutations associated with INH resistance in all INH resistant MTB strains and determined the association of mutations in the codon 315 of the *katG* gene and *inhA* mutations with clinical characteristics, drug resistant patterns, genotypes of the causative strains and treatment outcome. Both the 315 *katG* and *inhA* mutations were significantly associated with relapse in new patients treated by 2SRHZ/6HE. In addition, 315 *katG* mutations were associated with poly-drug resistance such as the combination of streptomycin, rifampicin and ethambutol. Although 315 *katG* mutations were not associated with treatment failure solely, these mutations were related to unfavorable treatment (treatment failure and death), as well as with Beijing genotype strains with resistance to streptomycin. This finding indicated that streptomycin resistance played an important role in the association between Beijing genotype and 315 *katG* mutations and increased probability of unfavorable treatment of 315 *katG* mutant strains. Therefore streptomycin should be discontinued in TB treatment regimen for new TB patients in Vietnam.

In Chapter 7, using isolates collected in a population-based study, we described that 4.1% of the strains circulating in a rural South of Vietnam lacked IS*6110* DNA. Therefore, the PCR assay to detect *M. tuberculosis* in clinical material that targets IS*6110*, which is widely used in laboratories in Vietnam, should be replaced with PCRs targeting other sequences, such as *rpoB* or IS*1081* to avoid missing TB cases of patients infected with strains lacking IS*6110*. In contrast to Beijing genotype, strains without IS*6110* were more sensitive to anti-TB drugs.

In Chapter 8, we compared the discriminative power of RFLP typing and 24 loci variable number of tandem repeat (VNTR) typing in clustering of 95 Beijing strains randomly selected from a population-based study. We found that 24 loci VNTR typing and RFLP typing had a similar, high level of discrimination among these strains with Hunter Gaston Discrimination Index was 0.994. Although loci VNTR 154, VNTR 2461 and VNTR 3171 hardly added any value to the level of discrimination, in comparison to 15 loci VNTR and RFLP typing, 24 loci VNTR typing revealed the highest level of discrimination among the 95 Beijing genotype isolates. For this and other practical reasons (VNTR typing is faster and easier to perform than RFLP typing), the last method is preferred in investigations on transmission of Beijing strains in Vietnam.

Chapter 9 discusses the findings of this thesis in the light of the literature and their meaning for tuberculosis control in Vietnam. It concludes that molecular epidemiology of *Mycobacterium tuberculosis* plays an important, indirect role in

studying the epidemiology of tuberculosis in this country and can support the National TB Program significantly.

TÓM TẮT

Luận án này mang đến một cái nhìn sâu sắc về sự phân bố các kiểu gen của vi khuẩn lao *Mycobacterium tuberculosis* tại một vùng nông thôn ở miền Nam Việt Nam, và mối liênhệ của chúng với sự đề kháng thuốc lao, với các đặc điểm lâm sàng và với kết quả điều trị. Nhờ có sự phối hợp của các phương pháp sinh học phân tử và dịch tễ học phân tử, luận án này đã chứng minh được thử nghiệm GenoType® MTBDR*plus* là một công cụ tốt để chẩn đoán lao đa kháng thuốc (MDR-TB), cũng như kiểu gen Bắc Kinh và sự đề kháng isoniazid là hai chỉ số dự đoán tình hình tái phát lao. Phát hiện này có thể chuyên biệt ở Việt Nam nơi mà những bệnh nhân lao được điều trị với phác đồ 8 tháng (2SRHZ/6HE); vì vậy cho nên người ta có thể dự đóan rằng phác đồ trên có thể là một trong những nguyên nhân gây ra tỷ lệ tái phát cao ở Việt Nam và nên được thay thế bằng phác đồ 6 tháng (2HRZE/4RHE) theo khuyến nghị của Tổ chức y tế thế giới. Hơn nữa, qua luận án này chúng ta cũng biết được, nhiễm hỗn hợp cùng lúc hai loại vi khuẩn lao không chỉ hiện diện ở những vùng có tỷ lệ bệnh lao cao, mật độ dân số cao và tỷ lệ nhiễm HIV cao, mà còn có mặt ở những nơi có tỷ lệ lao trung bình, tỷ lệ HIV thấp. Thêm vào đó, các chủng không có đoạn IS*6110* cũng gây khó khăn cho Việt Nam trong việc chẩn đoán lao cho các trường hợp âm giả khi xét nghiệm PCR *IS6110*.

Chương 1 giới thiệu tổng quan về gánh nặng và dịch tễ học của bệnh lao trên toàn thế giới và tại Việt Nam. Đồng thời, chúng tôi cũng giới thiệu về cấu trúc phân tử của vi khuẩn lao, và sơ lược những kỹ thuật phân tử dùng để đánh dấu gene và chẩn đoán bệnh. Mục đích của luận án cũng được mô tả trong chương này.

Những phát hiện trong Chương 2, sử dụng các số liệu từ cuộc khảo sát lao kháng thuốc toàn quốc (DRS) tại Việt Nam thực hiện trong giai đoạn 2004-2005, cho thấy thử nghiệm Genotype® MTBDR*plus* có độ nhạy và độ đặc hiệu cao, đồng thời các giá trị tiên đoán dương và âm có thể chấp nhận được trong chẩn đoán thường quy lao đa kháng (MDR-TB), với độ nhạy cảm và độ đặc hiệu lần lượt là 89% và 100%. Thêm vào đó, thử nghiệm GenoType® MTBDR*plus* cũng phát hiện được tình trạng nhiễm hỗn hợp của các chủng kháng và nhạy của vi khuẩn lao, hoặc hỗn hợp của vi khuẩn lao MTB và vi khuẩn lao không điển hình (NTM).

Trong chương 3, dựa trên hai phương pháp spoligotyping và Restriction Fragment Length Polymorphism (RFLP), chúng tôi đã đánh dấu các chủng *Mycobacterium tuberculosis* phân lập từ một nghiên cứu dựa trên dân số, và thấy ở nông thôn miền Nam Việt Nam, nơi có tỷ lệ lao năm 2005 là khoảng 100/100.000 dân số, có ít nhất 3,1% số bệnh nhân lao, đã bị nhiễm cùng lúc hai loại vi khuẩn lao. Nhiễm trùng kép như vậy thường là một hỗn hợp của một chủng Bắc Kinh với một chủng East African Indian, là hai kiểu gen phổ biến nhất ở Việt Nam. Trong nghiên cứu này chúng tôi thấy rằng nhiễm hỗn hợp không liên quan đến các triệu chứng lâm sàng và sự đề kháng thuốc. Tuy rằng, nhiễm hỗn hợp cũng ít xảy ra ở những bệnh nhân có tổn thương X quang ngực lan rộng và có đàm soi dương cao, điều này cho thấy những bệnh nhân nhiễm kép có khả năng đáp ứng miễn dịch gia tăng khi nhiễm lao.

Trong chương 4 và chương 5, chúng tôi đánh giá mối quan hệ giữa kiểu gen Bắc Kinh với tái phát hoặc thất bại điều trị dựa trên một nghiên cứu đoàn hệ tiến cứu. Trong chương 4, chúng tôi thấy rằng nguy cơ tương đối của tái phát sau khi điều trị khỏi đối với các chủng Bắc Kinh cao hơn so với các chủng khác (OR 5.5; 95% CI, 2,1-14,6). Tỉ lệ này cao hơn một nghiên cứu đa quốc gia trước đây của Bumman và cộng sự, trong đó

các bệnh nhân cũng được theo dõi lao tái phát chủ động. Tỉ lệ tái phát lao cao hơn trong các trường hợp nhiễm chủng Bắc Kinh, có thể liên quan đến việc sử dụng phác đồ điều trị 2SRHZ/6HE cho các bệnh nhân lao mới. Vì ethambutol là một thuốc kìm khuẩn, có thể ngăn cản vi khuẩn tăng trưởng nhưng không thể tiêu diệt chúng, do đó khi ngưng điều trị thuốc lao, các vi khuẩn còn sống sót sẽ tăng trưởng trở lại gây ra triệu chứng lao và cấy dương tính trong vài tháng sau đó. Tuy nhiên chủng Bắc kinh không liên quan trực tiếp đến tỉ lệ thất bại điều trị lao, như đề cập trong Chương 5.

Trong một nghiên cứu tiến cứu dựa trên dân số được mô tả, trong Chương 6, chúng tôi dùng thử nghiệm GenoType® MTBDR*plus* để phát hiện các đột biến kháng INH trong tất cả các chủng lao kháng isoniazid, sau đó xác định mối liên quan giữa các đột biến 315*katG* và *inhA* với các đặc điểm lâm sàng, kiểu kháng thuốc, kiểu gene của các vi khuẩn lao gây bệnh, và kết quả điều trị. Cả hai đột biến này đều liên quan mật thiếtđến lao tái phát ở bệnh nhân lao mới, được điều trị với phác đồ 2SRHZ/6HE, ngoài ra đột biến 315*katG* còn kết hợp có ý nghĩa với kháng nhiều thuốc như streptomycin, ethambutol và rifampicin, mặc dùđột biến 315*katG* không kết hợp với thất bại điều trị đơn thuần và chủng lao Bắc Kinh, nhưng đột biến này có liên quan đến kết quả điều trị kém (thất bại điều trị và tử vong), cũng như kết hợp có ý nghĩa với chủng lao Bắc Kinh khi chủng này kháng với streptomycin. Phát hiện này thừa nhận rằng kháng streptomycin đóng một vai trò quan trọng trong sự kết hợp giữa chủng Bắc Kinh và đột biến 315*katG* cũng như gia tăng khả năng đáp ứng điều trị kém của đột biến 315*katG*. Vì vậy nên ngừng sử dụng streptomycin trong phác đồ lao cho bệnh nhân lao mới tại Việt Nam.

Trong Chương 7, sử dụng các chủng thu thập từ nghiên cứu dân số, chúng tôi nhận thấy khoảng 4.1% chủng vi khuẩn lao thiếu đoạn IS*6110* (no-copy) đang lưu hành tại vùng nông thôn Việt Nam. Do đó thử nghiệm PCR dựa vào đoạn đích IS*6110,* đang được sử dụng rộng rãi tại các phòng xét nghiệm tại Việt Nam để phát hiện vi khuẩn lao trong các bệnh phẩm lâm sàng, nên được thay thế bằng các thử nghiệm PCR với đích là các trình tự khác như *rpoB* hay *IS1081* để tránh bỏ sót các trường hợp bệnh nhân nhiễm chủng lao thiếu đoạn IS*6110*. Khác với chủng Bắc Kinh, chủng no-copy thường nhạy cảm với thuốc kháng lao.

Trong chương 8, chúng tôi so sánh độ phân nhóm của phương pháp Restriction Fragment Length Polymorphism (RFLP) và 24 loci Variable Number of Tandem Repeat (VNTR). Trong số 95 chủng Bắc Kinh được chọn ngẫu nhiên từ một nghiên cứu dân số, chúng tôi nhận thấyphương pháp VNTR 24 locivà RFLP có độ phân nhóm cao tương đương nhau đối với chủng Bắc Kinh, vớichỉ số phân nhóm Hunter Gaston là 0.994. Tuy nhiên các locus VNTR 154, VNTR 246 và VNTR 3171 hầu như không có giá trị để phân nhóm các chủng Bắc Kinh. So với 15 loci VNTR thì RFLP và 24 loci VNTR có độ phân nhóm 95 chủng Bắc Kinh cao nhất. Vì lý do này và những lý do thực tế khác (VNTR thực hiện dễ hơn và nhanh hơn so với RFLP), cho nên phương pháp 24 loci VNTR được ưa thích hơn trong việc điều tra nguồn lâycủacác chủng Bắc Kinh tại Việt nam.

Chương 9, bàn luận về những kết quả phát hiện trong luận án này dựa theo tài liệu từ các nghiên cứu trước và về ý nghĩa của luận án đối với việc kiểm soát lao tại Việt Nam. Từ đó kết luận rằng dịch tễ học phân tử của vi khuẩn lao đóng một vai trò gián

tiếp quan trọng trong việc nghiên cứu dịch tễ học của bệnh lao tại Việt nam và có thể trợ giúp cho Chương Trình Chống Lao Quốc Gia một cách có ý nghĩa.

SAMENVATTING

Dit proefschrift biedt inzicht in de distributie van *Mycobacterium tuberculosis* genotypen in het rurale zuiden van Vietnam, en hun relatie met resistentie tegen anti-tuberculose middelen, klinische karakteristieken en behandeluitkomst. Dankzij de combinatie van moleculair-biologische- en epidemiologische methoden, toont dit proefschrift aan dat de GenoType® MTBDR*plus* test een betrouwbare methode is om multidrug resistente tuberculose (MDR-TB) te diagnosticeren. Verder zijn het Beijing genotype van *M. tuberculosis* en isoniazide resistentie twee voorspellers van recidieven bij tbc patiënten. Het is mogelijk dat deze effecten specifiek zijn voor de situatie in Vietnam waar eerste-lijnsbehandeling plaatsvindt met een 8-maanden schema (2SRHZ/6HE). Men zou kunnen speculeren dat dit schema één van de oorzaken is van het hoge percentage relapsen in Vietnam, en dat dit vervangen zou moeten worden door een 6-maanden schema (2HRZE/4RHE) in overeenstemming met de aanbevelingen van de WHO. Bovendien toont dit proefschrift aan dat multipele infecties van *M. tuberculosis* niet alleen aanwezig zijn in regio's met een hoge prevalentie van tbc, een hoge dichtheid aan bevolking en een hoge prevalentie van HIV. Tevens vormen stammen zonder de PCR target IS*6110* een probleem in Vietnam betreffende fout-negatieve moleculaire diagnose in de IS*6110* PCR.

Hoofdstuk 1 geeft een algemene introductie over de omvang van het probleem en de epidemiologie van tuberculose wereldwijd en in Vietnam. Tevens wordt hierin de populatiestructuur van *M. tuberculosis* in dit land geïntroduceerd, alsmede de moleculaire technieken voor genotypering en de diagnose. Bovendien worden de doelstellingen van dit proefschrift beschreven in dit hoofdstuk.

De bevindingen in Hoofdstuk 2, gebruikmakend van een nationale tbc resistentie survey in Vietnam die werd uitgevoerd in 2004-2005, geven aan dat de GenoType® MTBDR*plus* test een hoge sensitiviteit, specificiteit, en een acceptabele positieve- en negatieve voorspellende waarde heeft in de routinediagnostiek van meervoudig resistente tuberculose (MDR-TB), met een test sensitiviteit en specificiteit van 89% en 100%, respectievelijk. Bovendien toonde de GenoType® MTBDR*plus* test diverse meervoudige infecties aan van resistente en gevoelige stammen van *M. tuberculosis* en van nontuberculeuze mycobacteriën (NTM) en *M. tuberculosis*.

In Hoofdstuk 3, op basis van spoligotypering en restriction fragment lengte polymorfisme (RFLP) typering van *Mycobacterium tuberculosis* stammen die geïsoleerd waren bij een populatie-gebaseerde studie, vonden we dat minstens 3.1% van de tbc patiënten in het rurale Zuid-Vietnam, waar de tbc prevalentie ongeveer 100/100.000 mensen bedroeg in 2005, geconfronteerd waren met meervoudige infecties van *M. tuberculosis*. Zulke dubbele infecties waren vaak een mengsel van een Beijing- en een East African Indian stam; de twee meest voorkomende genotypen in Vietnam. In deze studie vonden we ook dat gemengde infecties niet geassocieerd waren met klinische symptomen en geneesmiddelenresistentie. Deze meervoudige infecties kwamen significant minder vaak voor bij patiënten met thoraxfoto's die uitgebreide afwijkingen vertoonden en met sterk positieve sputum microscopie, hetgeen suggereert dat patiënten met multipele infecties een verhoogde tolerantie hebben voor *M. tuberculosis* infecties.

In Hoofdstuk 4 en 5 bepaalden we de correlatie tussen het Beijing genotype en de kans op recidieven en falende behandeling, gebaseerd op een prospectieve cohort studie. In Hoofdstuk 4 vonden we dat het relatieve risico op recidief na curatieve behandeling groter was voor patiënten met Beijing- versus non-Beijing isolaten (OR 5.5; 95% CI, 2.1-14.6). Deze verhouding was meer uitgesproken dan eerder werd waargenomen in de meerlanden studie van Burman *et al.* waarbij patiënten werden gevolgd op het opnieuw ontwikkelen van tbc. Het hogere percentage van terugkerende tbc in ziektegevallen die veroorzaakt waren door Beijing stammen zou geassocieerd kunnen zijn met het gebruik van de 8-maanden 2SRHZ/6HE schema's in nieuwe patiënten. Omdat ethambutol een bacteriostatisch middel is, kan het wel de groei van bacteriën remmen, maar het kan ze niet doden. Dus wanneer de behandeling wordt gestopt, kunnen de overgebleven bacteriën weer gaan repliceren en binnen een paar maanden weer symptomen veroorzaken en positieve kweken. Alhoewel; het Beijing genotype op zich beïnvloedt het falen van de behandeling niet, zoals staat vermeld in Hoofdstuk 5.

In een prospectieve, populatie-gebaseerde studie die beschreven is in Hoofdstuk 6, gebruikten we de GenoType® MTBDR*plus* test om mutaties te detecteren die geassocieerd zijn met INH resistentie in alle INH resistente MTB stammen en bepaalden we de associatie van mutaties in het codon 315 van het *katG* gen en *inhA* mutaties met klinische karakteristieken, resistentiepatronen,genotypen van de causale stammen en behandeluitkomst. Zowel de 315*katG* als de *inhA* mutaties bleken significant geassocieerd met recidief in nieuwe patiënten die behandeld werden volgens het schema 2SRHZ/6HE. Verder waren 315*katG* mutaties geassocieerd met poly-drug resistentie, zoals de combinatie van resistentie tegen streptomycine, rifampicine en ethambutol. Alhoewel 315*katG* mutaties op zich niet geassocieerd waren met falende behandeling, waren deze mutaties wel gerelateerd aan ongunstige uitkomst (falende behandeling en sterfte gecombineerd), en ook Beijing genotype stammen met resistentie tegen streptomycine. Deze bevinding geeft aan dat streptomycine resistentie een belangrijke rol speelt in de associatie tussen het Beijing genotype en 315*katG* mutaties en toegenomen kans op ongunstige behandeluitkomst bij patiënten met 315*katG* mutantenstammen. Daarom zou het gebruik van streptomycine gediscontinueerd moeten worden in de behandeling van nieuwe tbc patiënten in Vietnam.

In Hoofdstuk 7, gebruikmakend van isolaten die verzameld waren in het kader van een populatie-gebaseerde studie, beschrijven we dat 4.1% van de stammen die in het rurale deel van Zuid-Vietnam circuleren geen IS*6110* DNA bevatten. Daarom zou de PCR methode om *M. tuberculosis* in klinisch materiaal aan te tonen en die gebaseerd is op de detectie van IS*6110* en die gebruikt wordt in veel laboratoria in Vietnam, vervangen moeten worden door PCR methoden die andere sequenties detecteren, zoals *rpoB* of IS*1081,* dit om te voorkomen dat er tbc gevallen gemist worden van patiënten die met stammen zijn geïnfecteerd die IS*6110* missen. In tegenstelling tot het Beijing genotype, zijn stammen zonder IS*6110* gevoeliger voor anti-tuberculose drugs.

In Hoofdstuk 8 hebben we het discriminerende vermogen onderzocht van RFLP typering en 24 loci variable number of tandem repeat (VNTR) typering bij 95 Beijing genotype stammen die random waren geselecteerd uit een populatie-gebaseerde studie.

We vonden hierbij dat 24 loci VNTR- en RFLP typering een vergelijkbaar, hoog niveau van discriminatie vertoonden, zoals bepaald met de Hunter Gaston Discrimination Index (0.994). Alhoewel de loci VNTR 154, VNTR 2461 and VNTR 3171 nauwelijks iets toevoegden aan het niveau van discriminatie, vertoonde in vergelijking met 15 loci VNTR- en RFLP typering, 24 loci VNTR typering het hoogste niveau van discriminatie onder de 95 Beijing genotype isolaten. Om deze en andere, meer praktische redenen (VNTR typering is sneller en gemakkelijker uit te voeren dan RFLP typering), is de 24 loci VNTR typering daarom eerste keuze voor onderzoek naar transmissie van Beijing genotype stammen in Vietnam.

Hoofdstuk 9 belicht de bevindingen in dit proefschrift in het kader van de literatuur en hun betekenis voor de tuberculosebestrijding in Vietnam. Er wordt geconcludeerd dat de moleculaire epidemiologie van tuberculose een belangrijke, zij het indirecte rol speelt in het bestuderen van de epidemiologie van tuberculose in dit land, en dat het daarom het nationale tbc-bestrijdingsprogamma in belangrijke mate kan ondersteunen.

ACKNOWLEDGEMENTS

The pathway leading me to come here today began in the winter 2003, when I was nominated to come to the Tuberculosis Reference Laboratory at RIVM in the Netherlands, for studying bio-molecular techniques to research the molecular epidemiology of *M. tuberculosis* in Vietnam. This study took eight years, from 2003-2011: it was sometimes smooth but many times unsteady on this route. Without the dedicated support of KNCV Tuberculosis Foundation, the National Institute of Public Health and the Environment (RIVM), the Amsterdam Institute for Global Health and Development (AIGHD) at the Academic Medical Centre of the University of Amsterdam, and Pham Ngoc Thach Hospital, I would not be able to present this thesis.
First of all I would like to honor:

- KNCV Tuberculosis Foundation and its Board of Directors for the financial support that gave me an opportunity to acquire new knowledge and new techniques in the field of *molecular epidemiology* of *Mycobacterium tuberculosis*
- RIVM and its Board of Directors where I learned these molecular techniques and acquired new knowledge, thanks to their help we completed these studies.
- AIGHD and its Board of Directors that dedicatedly helped me to accomplish this thesis as well as taught me statistical analyses.
- Pham Ngoc Thach Hospital and Board of Director created good conditions for me to finish this thesis.
- All patients who participated in this study.

I would like to send my warmest thanks to my five dedicated and modest teachers, who spent a lot of time to help me complete this thesis and have quietly made possible the extremely valuable research contributing to the progress of medicine. Teachers with conscience and noble careers will always be a bright example for me to follow. Please accept here my respect and warmest gratitude:

- Professor Dick van Soolingen, Head of Department of Pulmonary Diseases and Medical Microbiology, at the Radboud University in Nijmegen and Head of the National Tuberculosis Reference Laboratory at RIVM, who taught me the knowledge and techniques of molecular biology and gave me many excellent ideas and precious comments to complete the papers as well as guided me the way to write this thesis.
- Professor Frank Cobelens, at the University of Amsterdam, who gave me the opportunity to take part in this PhD program in the Netherlands and taught me the statistical analyses. He gave me precious comments and spent a lot of time with me to analyze and finish the papers as well as guided me the way to write this thesis.
- Doctor Edine Tiemersma, working at KNCV, who taught me the basics of biostatistics and study design. She has supported me during most of the time in the Netherlands and gave me the opportunity to participate in courses and conferences to enhance my knowledge. She guided me step by step and gave me excellent comments to accomplish this thesis.
- Doctor Kristin Kremer, working at World Health Organization, in Denmark. She taught me the basic techniques of molecular biology and went along with me

during the time I studied at the RIVM and gave me new molecular knowledge that I had never known. She gave me valuable comments and corrections to complete the papers of this thesis.

- ❖ Doctor Nguyen Thi Ngoc Lan, Head of microbiology department in Pham Ngoc Thach Hospital, who led me on the path of scientific research and created favorable conditions for me to complete this thesis. She taught me the basics of research design and traveled with me during the past 20 years of scientific research.

I would like to send my deeply felt thanks to:

- ❖ Doctor Nguyen Huy Dung, Director of Pham Ngoc Thach Hospital, who wholeheartedly helped me and created favorable for me to complete this thesis.
- ❖ Doctor Pham Duy Linh and Doctor Hoang Thi Quy, former Directors of Pham Ngoc Thach hospital, who helped and created favorable conditions for me to participate in scientific research.
- ❖ The Tien Giang provincial TB program that dedicatedly helped to collect and monitor patients and created favorable conditions to complete this study.
- ❖ The Vietnamese National TB program that created favorable conditions for me to acquire new techniques.

I would like to send my warmest thanks to:

- Anne-Marie van den Brandt, who first welcomed me at RIVM, who has instructed me on every technical detail and accompanied me in the early days, when I accustomed myself to new techniques. She spared a lot of time and tried all her best to help me successfully perform the molecular biological techniques.
- My best friend Phan Thi Hoang Anh, who has been working with me since the first days when the molecular techniques were developed in our lab. She was with me going through the most difficult days for my success today. With her meekness and honesty she has been quietly helping us complete the invaluable contribution to medical progress.
- Mirjam Dessens, who spared no effort to teach me step by step in working with the Bionumerics program in the first time I visited RIVM.

I would like to send my sincere gratitude to:

- ➢ Doctor Tran Ngoc Buu and his colleagues in Pham Ngoc Thach National TB control Program who have helped me to gather valuable data as well as supported me in statistical analysis. Thanks to their help this thesis is completed.
- ➢ Dai Viet Hoa, former vice Head of microbiology lab, and her colleagues have performed TB culture and drug susceptibility testing; they have been quietly helping us complete the invaluable contribution to medical progress. It is thanks to their results that we have finished this study.

- Doctor Maxine Caws and her staff at the Oxford University Clinical Research Unit in Ho Chi Minh City for creating good opportunity for us to work with their sequencer, thanks to her dedicated help we accomplished this study.
- Co-authors Petra de Haas, Doctor Rick O'Brien and Professor Christopher Sola who gave me precious comments to accomplish the papers.
- The staff of the Mycobacteriology Department at the RIVM is gratefully acknowledged for helping me to complete my study.
- Arnout Mulder, Jessica de Beer, and Mimount Enaimi who spared no effort to help me analyze the VNTR results and optimize the figures.
- Doctor Arnold Herrewegh, who helped me to get my visa, found good places for me to stay and repaired the bike for my driving in the Netherlands.
- Tridia van der Laan, Miranda Kamst and Rina de Zwaan are deeply thanked for helping me everything during the time I have studied in RIVM.
- Ms Truong Thi Kim Hue, former supervisor of microbiology department, who well arranged all needed equipment and chemicals ready to carry out the research.
- Doctor Michael F. Iademarco and Doctor Deborah Dean, who taught me the basics of Epi-info, molecular biology techniques and gave me the opportunity to study in the USA.
- Professor Nguyen Thanh Bao, Doctor Bo Thi Kim Khanh, Doctor Pham Hung Van, Doctor Nguyen Thi Chi Mai, Doctor Bui Nang Thien, who brought the shine of micro and molecular biology to my knowledge.

I also send my honest thanks to Ms Gera Wolswijk, Serena Stockmann and Emma R. Veenstra, who gave me warm places to stay during my study in the Netherlands.

I would like to thank Ms Tran Kim Phung, Ms Carla Lanen in support me in my visa documents.

I am much obliged to Doctor Pham Thu Hang, Ms Tran Thi Kim Quy and Microbiology lab in Pham Ngoc Thach hospital, where I have had a strong attachment to all my colleagues for 20 years, for your sharing joys and sadness of life. All of you have silently supported me to complete this thesis.

With the love of humanity, I would like to send the patients: a blessing for those who are still alive and a prayer for those who died.

Finally, I would like to send my warmest thanks with sincerity to my parents, who have sacrificed their life for my success today and to my brothers and sisters who have quietly helped me to complete my study.

CURRICULUM VITAE

Mai Nguyệt Thu Huyền was born on 4 November 1967 in Saigon, in Southern Vietnam. She grew up with her parents and her siblings in this city. When she was 7 years old she dreamt of becoming a singer, but her dream did not come true due to the war. After the war, she changed her mind and chose medicine for her career.

She passed school exams and entered the University of Medicine in Ho Chi Minh City in 1986, and graduated as a General Physician in 1992. After graduation, she accepted a position in Pham Ngoc Thach Tuberculosis and Lung Disease Center (later named Pham Ngoc Thach Hospital) in the Microbiology Department. She acquired further training in microbiology during a one-year course at the University of Medicine in Ho Chi Minh City and graduated as a medical microbiologist in 1993. She has formally worked in the non *Mycobacterium tuberculosis* laboratory in Pham Ngoc Thach hospital since 1993.

In 1999, she got a scholarship of WHO for a one-month course on *Mycobacterium tuberculosis* in South Korea. One year later, she went to the USA to study diagnostic techniques of Chlamydia infection and PCR.

In 2002, she attended to a short course in Vietnam on analysis of molecular typing data organized by RIVM and Pham Ngoc Thach Hospital. This was the start of extensive training in bio-molecular techniques (RFLP, spoligotyping, VNTR, Bionumerics, Genemarker) during several extended visits to RIVM in the period 2003-2011, as a basis for her research of the transmission dynamics of tuberculosis in a rural area of Vietnam, and an important turning point in her life.

In 2009, with dedicated and kind supports from KNCV Tuberculosis Foundation and RIVM, she received a grant to keep studying as a PhD candidate from 2009-2013. This helped her to build upon her professional strengths and to increase her efficiency in weaker areas. Her study focused on molecular epidemiology of tuberculosis since 2009 in Vietnam.